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Regulation of alpha-actin gene expression during BC3H1 myogenic cell differentiation

Reeser, Jonathan Charles, Ph.D.

The Ohio State University, 1991
REGULATION OF ALPHA-ACTIN GENE EXPRESSION DURING BC3H1 MYOGENIC CELL DIFFERENTIATION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Jonathan Charles Reeser, B.S.

*****

The Ohio State University

1991

Dissertation Committee:
Robert M. DePhilip, Ph.D.
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Arthur R. Strauch, Ph.D.

Approved By:

Advisor
Department of Anatomy
"Each one of us adds a little to our understanding of Nature, and from all the facts assembled arises a certain grandeur."

*Aristotle*

"All men dream: but not equally. Those who dream by night in the dusty recesses of their minds wake in the day to find that it was vanity: but the dreamers of the day are dangerous men, for they may act their dream with open eyes, to make it possible."

*T. E. Lawrence*

"Assembly of Japanese bicycle require great peace of mind."

*Robert M. Pirsig*

"You've got to pay your dues if you want to sing the blues, and you know it don't come easy."

*Ringo Starr*
To Nandita
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It is with deep affection and sincere appreciation that I acknowledge the kind assistance and moral and technical support of the people who have guided me towards my goal of obtaining a doctorate degree in Cell Biology, Neurobiology, and Anatomy.

I am grateful to my adviser, Dr. Arthur R. Strauch, for accepting me into his laboratory and for allowing me to investigate the cellular and molecular biology of actin gene regulation. His unique brand of humor and his willingness to let me explore unfamiliar territory were appreciated and will be long remembered.

I am indebted to the members of my general exam and dissertation committees for their time and energy. Dr. Robert M. DePhilip, Dr. John M. Robinson, and Dr. Roy A. Tassava are to be commended for their strong commitment to graduate education. I must also express my gratitude to Dr. Richard W. Burry for his interest and concern.

I offer my thanks and best wishes to several student and postdoctoral colleagues who made the long and occasionally fruitless hours in the laboratory more enjoyable. Mark D. Berman, Howard R. Miller, Daniel G. Danahey, James J. Lah, and Dr. Hua Yan were always eager to share information, or simply conversation, and I am honored by their friendship.

My thanks and best wishes also go to Dr. Douglas and Linda Foster, who supplied plentiful stocks of several clones as well as useful technical advice, and to Chris Klein and Dr. Arthur Burghes, who assisted me in debugging the Southern blot protocol.

I am grateful to my parents, Robert M. and Pauline F. Reeser, for instilling in me a love of learning, and to my parents-in-law, Narayan R. and Ingrid U. Bhattacharjee, for their support during my graduate and professional education. Finally, and most importantly, I must acknowledge my wife, Nandita Bhattacharjee. Her assistance, advice, encouragement, and patience have been of immeasurable benefit to me during my pursuit of the M.D. and Ph.D. degrees, and it is to her that this work is dedicated.
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LIST OF ABBREVIATIONS

CA : Cardiac
CAT : chloramphenicol acetyl transferase
CPM : Counts per minute
ddH₂O : Double distilled water
DEPC : Diethylpyrocarbonate
DMEM : Dulbecco’s Modified Eagle’s Medium
DNA : Deoxyribonucleic Acid
EDTA : Ethylenediamine Tetraacetic Acid
EtBr : Ethidium Bromide
FBS : Fetal bovine serum
g : Gravitational force constant
GTG : Genetic Technology Grade
HEBS : HEPES buffered saline
HEPES : N-[2-Hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid]
HSB : High salt buffer
µCi : Microcuries
µg : Micrograms
µl : Microliters
mRNA : messenger Ribonucleic Acid
mg : Milligrams
ml : Milliliters
MOPS : 3-[N-Morpholino] propane-sulfonic acid
ng : Nanograms
nm : Nanometers
PBS : Phosphate buffered saline
PMSF : Phenylmethylsulfonyl flouride
RNase : RNAse
TAE : Tris Acetate EDTA
TBS : Tris Buffered Saline
TCA : Trichloroacetic Acid
TE : Tris EDTA
TES : [N-tris(hydroxymethyl)methyl-2-aminoethylsulfonic acid] EDTA SDS
TLC : Thin Layer Chromatography
TLE : Thin Layer Electrophoresis
Tm : Melting temperature of a nucleic acid duplex
SAM : Substrate attached material
SDS : Sodium Dodecyl Sulfate
SK : Skeletal
SSC : Saline Sodium Citrate
VSM : Vascular smooth muscle
GENERAL INTRODUCTION

One of the central problems in cell and developmental biology is to understand the regulation of stage-specific gene expression during development. Myogenesis is a useful and popular system for studying developmental regulation, since muscle-specific proteins appear in temporally coordinated sequences during differentiation. Expression of the contractile protein actin during myogenesis is characterized by "isoform switching", wherein the two actin isoforms expressed in undifferentiated muscle cells are largely replaced during muscle differentiation and maturation by one or more muscle-specific actin isoforms. The experiments I performed as part of my graduate research training and which comprise this dissertation were all designed to improve our understanding of the epigenetic mechanisms which regulate actin isoform switching during differentiation of mammalian myogenic cells in vitro.

Actin is the major component of the eukaryotic contractile apparatus and cytoskeleton and is involved in a variety of essential cellular processes and functions including cell locomotion, cell adhesion, maintenance of cell shape, force generation and
contractility, and signal transduction (Alberts et al. 1989; Pienta et al. 1989). Within the cell, actin interacts with a multitude of associated proteins that function as regulators of actin polymerization and which act to cap, bundle, and sever F-actin filaments in addition to controlling other actin-protein interactions (Pollard 1986; Stossel et al. 1985; Weeds 1982). Amino acid sequence analysis has revealed that there are at least six different actins expressed in higher mammals (Korn 1982; Vandekerckhove and Weber 1978a; Vandekerckhove and Weber 1978b; Vandekerckhove and Weber 1979; Vandekerckhove and Weber 1981); a seventh actin isoform has been isolated from and is apparently unique to chicken muscle cells (Bergsma 1985). These six actins have each been given descriptive names based on their isoelectric focusing gel separation behavior and on their principal tissue distribution in either muscle or nonmuscle cells. β- and γ-actin have relatively basic pl's, and are also referred to as nonmuscle or cytoplasmic actins because they are the only actins expressed in nonmuscle cells. Cardiac (CA), skeletal (SK), and vascular smooth muscle (VSM) α-actin isoforms, also referred to as α-actins or muscle-specific actins, are more acidic than the nonmuscle actins. In addition, there is also a γ-actin which is found in enteric smooth muscle.

All actins are highly conserved; even the most divergent actins, β- actin and SK α-actin, demonstrate a remarkable 93% primary sequence homology. Among the muscle actins, SK α and CA α differ by only four amino acid residues out of 375. SK and VSM α-actins differ by only eight residues; CA and VSM α-isoactins differ by only six amino
acid residues. Significantly, the majority of these primary sequence differences are clustered in the highly acidic amino terminal end of the actin molecule. The lack of evolutionary divergence as a whole and the localized primary sequence differences which distinguish the actin isoforms suggest not only that the entire actin molecule is functionally important but that in addition the amino terminal domain may mediate presumed tissue-specific interactions with associated molecules. Indeed, chemical cross-linking experiments have shown that the amino terminal of actin contains the binding site for myosin heavy chain (another developmentally regulated protein belonging to a multi-gene family) (Sutoh 1983), as well as the actin-binding proteins α-actinin, depactin, and fragmin (Buckingham 1985; Pollard 1986; Sutoh and Hatano 1986; Sutoh and Mabuchi 1984).

Actin genes from a variety of species have been cloned, and DNA sequence analysis has revealed a significant conservation of intron/exon arrangement among all actin genes in the deuterostoma (Buckingham 1985; Carroll et al. 1986; Engel et al. 1981). The 5' flanking promoter regions of all the α-actin genes contain the TATA consensus sequence, CC(A/T)\_6GG (CArG box) motifs, and CANNTG (E box) elements, and recent experiments have begun to identify those upstream regulatory sequence motifs which govern cell and tissue specific transcription of these genes (Carroll et al. 1988; Wade and Kedes 1989). Interestingly, regulation of the three muscle α-actin genes is strikingly different. The 3' untranslated regions of most actin genes are isotype-specific and are evolutionarily conserved (Ponte et
al. 1983), although the 3' UT of the VSM α-actin gene is not conserved between species (Carroll et al. 1986). These observations complement the amino acid sequence data, and reinforce the speculation that actin isoform conservation and tissue specific expression relate to specific functional roles during development and differentiation.

The development of actin gene-specific probes has permitted a detailed examination of actin isoform expression during muscle development. In 1982, Minty et al reported that a gene cloned from a fetal calf skeletal muscle cDNA library was in fact homologous with the cardiac α-actin gene (Minty et al. 1982). It is now well established that CA α-actin is the major actin gene expressed in fetal skeletal muscle, whereas SK α-actin predominates in adult skeletal muscle (Vandekerckhove et al. 1986). Such actin "isoform switching" is a general feature of muscle development in vitro and in vivo; skeletal α-actin is co-expressed with cardiac α-actin during cardiogenesis (Sassoon et al. 1988) and expression of nonmuscle β- and γ-actins is down-regulated concomitant with up-regulated expression of VSM α-actin during vascular smooth muscle development (Schwartz et al. 1986). In addition, it was recently reported that VSM α-actin is transiently expressed early in avian cardiogenesis (Ruzicka and Schwartz 1988) and in embryonic rat skeletal and cardiac muscle (Sawtell and Lessard 1989; Woodcock-Mitchell et al. 1988).

These patterns of actin gene expression during muscle development are of significance not only for their implications of the existence of unique isoactin functions during myogenesis, but because muscle cells involved in some disease processes will frequently
undergo phenotypic modulation and re-express the fetal pattern of actin isoforms. For example, smooth muscle proliferation in the blood vessel wall is an important factor in the pathogenesis of arteriosclerosis and hypertension (Campbell and Campbell 1985; Davies 1986; Munro and Cotran 1988; Ross 1986; Schwartz and Reidy 1987); proliferating vascular smooth muscle cells in vivo express reduced levels of VSM α-actin and increased levels of nonmuscle actins (Kocher and Gabbiani 1986; Kocher and Gabbiani 1987). Cardiomyopathies, such as congestive heart failure, are characterized by cardiomyocyte hypertrophy and by increased expression of the "fetal" skeletal α-actin isoform (Bishopric et al. 1987; Izumo et al. 1988; Parker et al. 1990). Upon resolution of the disease process the isoactin pattern typically returns to that of the differentiated phenotype (Kocher and Gabbiani 1987). Although a great deal of research has been devoted to investigating the mechanisms of cell phenotypic modulation, with particular emphasis on the action of growth factors and mitogens on vascular smooth muscle cells (reviewed in Florini and Magri 1989; Corjay et al 1990; Liau, 1990), the significance of "reverse isoactin switching" during muscle diseases and its role in the disease process is unknown.

Although much of the work characterizing α-actin gene expression in healthy and diseased muscle has been done in animals or utilized primary cultures of muscle cells (Schubert 1984; Schwartz et al. 1986; Schwartz and Reidy 1987), there also exist several muscle cell lines which permit us to examine and experimentally manipulate myogenic differentiation in vitro. These clonal cell lines preserve
many of the essential biochemical and morphological features of embryonic muscle development, while providing the advantage of cellular homogeneity and convenient growth and biochemical analysis in culture. For example, morphological and biochemical differentiation in the mouse BC3H1 myogenic cell line is inducible and fully reversible. Proliferating undifferentiated BC3H1 myoblasts in serum-containing media express predominantly nonmuscle β- and γ-actins, whereas postconfluent fully-differentiated myocytes in serum-free media down-regulate nonmuscle actin isoform expression and up-regulate α-actin expression. The BC3H1 cytodifferentiation process is arrested and reversed by replating differentiated cells at subconfluent densities in serum-containing medium. The BC3H1 cell line therefore appears to be a good experimental system in which to examine the dynamic regulation of α-actin gene expression during myogenesis and also during phenotypic modulation that accompanies the dedifferentiation process.

The studies described in this dissertation suggest that the sequential expression of two α-actins during BC3H1 myogenic cell differentiation is regulated at least in part at the level of gene transcription, and that this regulation is mediated largely by epigenetic signals from the microenvironment rather than by preprogrammed developmental switches. In addition, the studies help to further characterize the BC3H1 myogenic cell line and demonstrate its usefulness in studies of multiple actin expression and utilization during myogenesis. The experimental results are presented in three separate sections. In Chapter I, evidence is presented which
demonstrates that BC3H1 cells sequentially express both VSM and SK 
α-actins during differentiation. The experiments described in Chapter 
II were designed to investigate the regulation of α-actin expression in 
BC3H1 cells. Finally, experiments which investigated the cis/trans 
mechanisms controlling transcription of the mouse VSM α-actin gene 
are described in Chapter III.
CHAPTER I

INTRODUCTION

Muscle development is a multi-stage process, the first step of which occurs when totipotent mesodermal stem cells undergo commitment to the myogenic lineage. Under appropriate conditions, myoblasts will differentiate into fetal muscle cells and subsequently mature into adult muscles. This general paradigm describes the ontogeny of all three forms of muscle found in vertebrates: smooth muscle, cardiac muscle, and skeletal muscle, although far more is known about skeletal muscle development compared to the other two muscle tissue types. While our understanding of the specific signals and factors which determine whether a mesodermal stem cell becomes a cardiomyoblast versus a skeletal myoblast is rudimentary at best, the unifying hallmark of muscle differentiation in general is the phenomenon of contractile protein "isoform switching".

Isoform switching describes the process by which the expression of muscle-specific protein isoforms is modified to meet the needs of an increasingly more specialized cell. Examples of structural
and regulatory proteins which have muscle-specific isoforms include tropomyosin, troponin T,C, and I, and myosin heavy and light chains (Buckingham 1985; Hallauer et al. 1987; Breitbart et al. 1985; Toyota and Shimada 1983). Actin isoform switching also occurs during both smooth muscle and sarcomeric muscle development. During skeletal myogenesis, vascular smooth muscle (VSM) and cardiac (CA) α-actin are expressed in myoblasts but are largely replaced by skeletal (SK) α-actin in mature skeletal muscle (Gunning et al. 1983a); during cardiogenesis, CA α-actin becomes the dominant actin isoform expressed in the developing heart, replacing SK α-actin (Gunning et al. 1983a; Ruzicka and Schwartz 1988).

Unfortunately, the physiological significance of actin isoform switching during myogenesis in vivo is not well understood. While the clustered amino terminal sequence differences that distinguish the isoactins and the developmentally regulated, tissue-specific expression of the various isoactins indeed implies that they perform unique roles during muscle differentiation, little conclusive data exists regarding the functional segregation of actin isoforms in various cell and tissue types. Immunocytochemical studies have suggested that functional sorting of actin isoforms does occur in myogenic cells (DeNofrio et al. 1989; Otey et al. 1988), and studies with muscle:nonmuscle heterokaryons have shown a correlation between the induction of muscle actin genes and cytoskeletal remodeling in the fused nonmuscle cell (Blau 1989; Miller et al. 1988). In addition, studies by Leavitt's group have demonstrated that smooth muscle α-actin is preferentially lost from transformed rodent fibroblasts (Leavitt et al. 1988).
1985), and that elevated synthesis of a mutant nonmuscle β-actin promotes tumorigenesis in some transfected fibroblast cell lines (Leavitt et al. 1987 a,b). Conversely, one recent study of the abnormal developmental regulation of CA and SK α-actins in BALB/c mice detected no obvious effects of altered isoactin expression on cardiac development or function (Garner et al. 1986). Finally, Gunning et al (Gunning et al. 1984) demonstrated that human CA α-actin accumulates in transfected mouse nonmuscle cells and associates with the nonmuscle cytoskeleton. Although this result argues against functional sorting of actin isoforms, the experimental design did not address whether CA α-actin monomers were self-associated into F-actin filaments or copolymerized with pre-existing nonmuscle actins. Indeed, Yang et al (1979) showed that copolymers of muscle actin and Acanthamoeba actin behave as a unique species of actin, and not as a mixture of homopolymers, suggesting that F-actins of varying isoform composition in fact exhibit functional differences in vivo. Taken together, these studies suggest but do not prove that the different isoactins perform unique functions in an isoform dependent manner.

Three different isoactins are expressed during normal vascular smooth muscle development. Nonmuscle β- and γ-actin are expressed at high levels in immature vascular smooth muscle cells, while mature vascular smooth muscle myocytes in the blood vessel wall express predominantly VSM α-actin. Kocher and Gabbiani (1986) determined that the ratio of α-actin to the combined level of β- and γ-actins expressed in vascular smooth muscle from five day old rats was
approximately 1, while the same ratio in six week old rats was roughly 4. This pattern of α-actin expression can be reversed during smooth muscle proliferative diseases, such as arteriosclerosis (Gabbiani et al. 1984). Compared to vascular smooth muscle from normal tunica media, the level of α-actin in smooth muscle obtained from atheromatous plaque had decreased from 60% to 10%, while the level of β-actin had increased from 40% to 70%. However, upon re-endothelialization and resolution of the vascular injury (Gabbiani et al. 1984), or by administering intra-arterial heparin (Clowes et al. 1988), the levels of the various actins returned to normal. These observations suggest that VSM α-actins may play a unique functional role in differentiated smooth muscle cells, and that VSM α-actin expression is actively repressed and perhaps somehow inappropriate in proliferating smooth muscle cells.

From a teleological perspective, the studies describing isoactin expression during vascular smooth muscle development and during arteriosclerosis make perfect sense if we assume that nonmuscle actins are utilized for nonspecific, undifferentiated cytoskeletal functions and that VSM α-actin is used only in smooth muscle cell-specific functions such as force generation. Although conducive to building models and hypotheses that explain the significance of VSM α-actin expression during smooth muscle cell development, such rigid "off/on" assumptions about nonmuscle and muscle actin function are not helpful in constructing models explaining α-actin expression during skeletal and cardiac muscle development because the sarcomeric muscles coexpress muscle-specific α-actins in the course
of normal differentiation in vivo. However, it is possible that subtle cellular adaptations which may accompany actin isoform switching during striated muscle development have escaped detection using currently available analytical tools and techniques.

In contrast to smooth muscle, the expression of muscle-specific α isoactins during skeletal muscle development in vivo has been well characterized and follows a predictable temporal sequence. For example, in total RNA isolated from day 10/11 chick embryonic hindlimb muscle, nonmuscle β- and CA α-actin mRNA are the dominant actin transcripts (Hayward and Schwartz 1986). After myoblast fusion and muscle innervation between days 13-16, the level of nonmuscle β- and CA α-actin mRNA drops to less than 10% of total actin mRNA and the level of SK α-actin mRNA increases to comprise over 90% of total actin mRNA. Coexpression of SK and CA α-actin mRNAs has also been observed in both embryonic and adult cardiac muscle (Sassoon et al. 1988). In the normal adult mouse heart, SK α-actin mRNA comprises roughly 2% of the total actin mRNA, while in diseased adult human heart approximately 50% of the total actin mRNA is of the SK α-actin isotype (Gunning et al. 1983a). Thus, diseased cardiac muscle follows the pattern of inducing expression of "fetal" actin isoforms during disease processes or injury, but our intuitive understanding of the different functions that might be performed by "fetal" and "adult" actin isoforms in developing sarcomeric muscle is somewhat confounded by the fact that both the fetal and the adult isoforms represent muscle-specific isotypes.
Much of the recent work into the regulation of actin isoform switching in muscle development has been performed in vitro, using primary cultures of aortic smooth muscles or embryonic skeletal myoblasts. Although primary muscle cell cultures may seemingly overcome the problem of cellular heterogeneity inherent in tissue/organ culture and immunohistochemistry, primary cultures of smooth muscle cells vary in their pattern of α-actin expression (Barja et al. 1986; Gabbiani et al. 1984; Kocher and Gabbiani 1986). For example, species and age differences all apparently influence VSM α-actin expression in vitro (Campbell et al. 1988), and with extended time in culture smooth muscle myocytes tend to irreversibly modulate into myoblastoid cells that are incapable of inducing VSM α-actin expression at confluence (Barja et al. 1986; Kocher and Gabbiani 1987). Additional work by Hayward and Schwartz (1986) on striated muscle cells also underscores the variable regulation of α-actins in vitro compared to in vivo. In primary cultures of muscle cells isolated from day 10/11 embryonic chick hindlimb, the level of CA α-actin mRNA remains greater than that of SK α-actin mRNA even after fusion. Thus, α-actin expression during skeletal myogenesis in vitro differs from what is observed in vivo, and may depend on variables such as culture conditions, tissue source of the muscle cells, and the stage of myogenic commitment at the time of culture (Hayward and Schwartz 1986).

To avoid the problems of primary culture reproducibility, our laboratory uses the mouse BC3H1 myogenic cell line as a model system for studying actin isoform switching during myogenesis. Strauch and
Rubenstein have previously presented data which suggested that post-confluent BC3H1 myoblasts may express a VSM α-actin (Strauch and Rubenstein 1984a,b; Strauch et al. 1986). The peptide mapping studies presented here indicate that late stage BC3H1 myocytes additionally express a sarcomeric α-actin polypeptide. Hybridization analysis of total RNA isolated from late stage BC3H1 myocytes with isoform-specific probes suggests that this sarcomeric actin corresponds to the SK α-actin isoform (Strauch and Reeser 1989). The coexpression of smooth and skeletal muscle α-actins in BC3H1 myogenic cells is unique and suggests that this cell line will be extremely useful for studying positive and negative transcriptional controls governing actin isoform switching during myogenesis in vitro. BC3H1 cells may also permit experimental examination of the functional significance of multiple isoactin expression during muscle development.
MATERIALS AND METHODS

CELL CULTURE METHODS

BC3H1 cell stock cultures were obtained from Dr. David Schubert of the Salk Institute, La Jolla, California. Cells were maintained in logarithmic stage growth by cultivation in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, (unless otherwise noted, all cell culture reagents were obtained from Gibco Lifetechnologies Inc., Grand Island, NY). The cells were grown at 37°C in a 5% CO₂ atmosphere, and were re-fed with fresh medium every three or four days. Cells were subcultured when they became 40-60% confluent. Post-confluent BC3H1 myoblasts were induced to differentiate by cultivation for up to six days in N2 hormone-supplemented, serum-free medium, (Bottenstein and Sato 1979; Strauch and Rubenstein 1984a) containing RPMI 1640, 5 μM bovine serum albumin, (Pentex™, fatty-acid free, Miles Laboratories, Naperville, IL), 5 μg/ml insulin, 100 μg/ml transferrin, 20 mM progesterone, 100 μM putrescine, 30 nM sodium selenite, 10 mM
4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (all from Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Fungizone. Alternatively, post-confluent myoblasts were allowed to differentiate by prolonged treatment with DMEM containing either 0.5% FBS or 10% FBS (DMEM 0.5% FBS or DMEM 10% FBS, respectively).

To examine cell proliferation, cells were grown in 35 mm dishes and at appropriate times either harvested by enzymatic digestion for determination of cell numbers, or assayed for incorporation of tritiated thymidine. Two µCi of [3H]thymidine (5-20 Ci/m mole, Amersham, Arlington Heights, IL) was added to each 35mm dish, and the cells incubated at 37° C for an additional 2 hours. Following cell lysis in 0.1% sodium dodecyl sulfate (SDS), isotope incorporation was measured in trichloroacetic acid (TCA) precipitates by liquid scintillation counting.

For actin labeling studies, cells were radiolabeled for 8 to 12 hours at 37° C with 60 µCi/ml L-[35S]cysteine or L-[35S]methionine (1200 Ci/mmol, Amersham) using either cysteine- or methionine-free serum-free N2 medium. Actin monomers were purified from cell extracts by DNase I affinity column chromatography, as described later.

Cells were visualized using an Olympus inverted phase contrast microscope, and photographed using an Olympus camera on Kodak TMAX film, ASA 400.
ACTIN ISOLATION AND PEPTIDE MAPPING

BC3H1 cells in the desired state of differentiation were radiolabeled with L-[35S]methionine or L-[35S]cysteine as described under Cell Culture methods, harvested by scraping at 0-4°C, and collected by centrifugation at 360 x g for 10 minutes. The cell pellet was sonicated in 4 ml of ice cold actin depolymerization buffer containing 0.75 M guanidine hydrochloride (Eastman Kodak, Rochester, NY), 0.5 M sodium acetate, 10 mM Tris-HCl, 0.5 mM calcium chloride, 0.5 mM ATP, 0.5% Triton X-100 (Sigma), and 1 mM phenylmethylsulfonyl flouride (PMSF), pH 8.0) (Strauch and Reeser 1989; Strauch and Rubenstein 1984a). The cell homogenate was passed a total of five times over a single DNase I (Type D, Worthington Biochemical Corporation, Freehold, NJ) "Affigel-10" or "Affigel-15" (Bio Rad, Richmond, CA) agarose affinity column (1 ml bed volume). Unbound protein was removed from the column by washing with 10 bed volumes of actin depolymerization buffer, and the bound actin was eluted with five bed volumes of a buffer containing 3 M guanidine hydrochloride, 0.5 M sodium acetate, and 2 mM calcium chloride. The eluate was dialysed against three 4L changes of distilled water at 4°C over a 24 hour period, then lyophilized to dryness. Tryptic digestions were performed by first oxidizing the cysteine sulfhydryl groups in actin with freshly prepared performic acid (Hirs 1956), then digesting with 40 μg/ml tosylphenylalanylichloromethyl ketonetryptsin (Worthington) in 0.1 M ammonium bicarbonate for 2 hours at 22°C (Strauch and Rubenstein 1984b; Vandekerckhove and Weber
Radiolabeled peptides were lyophilyzed to dryness, suspended in pyridine acetate buffer, pH 6.3 (pyridine/acetic acid/water, 25:1:225) and spotted onto a 20 cm x 20 cm 160 µm thick cellulose thin layer electrophoresis plate, (without fluor, Eastman Kodak). The tryptic peptides were electrophoretically separated at 400 volts constant voltage in the same pyridine-acetate buffer system using Orange G as a marker dye. Autoradiographs were prepared by exposing dry thin layer plates to XAR-5 x-ray film (Kodak) for periods ranging from 6 hours to two weeks. Selected amino-terminal tryptic peptides were eluted from the cellulose with water, lyophilized, dissolved in pyridine acetate buffer, pH 3.3 (pyridine/acetic acid/water, 1:25:225), then electrophoretically separated at 400 volts constant voltage in the same buffer system using xylene cyanol FF as the marker dye. Eluted amino terminal tryptic peptides were further characterized by secondary cleavage with thermolysin (Boehringer Mannheim, Indianapolis, IN) or *Staphylococcus aureus* V8 protease (Miles) (Vandekerckhove and Weber 1981). Thermolysin digestions were performed at 37° C for 30 minutes in 0.2 M ammonium acetate, 5 mM calcium chloride pH 8.5 buffer using 1 µg thermolysin in 100 ml buffer and then spotted directly onto cellulose thin layer electrophoresis plates. V8 protease digestions were performed at 37° C for 20 hours in 0.5% ammonium bicarbonate using 5 µg enzyme per 100 ml buffer; reaction mixtures were then lyophilized to remove the ammonium bicarbonate prior to electrophoresis at pH 6.3.
PREPARATION OF GENE SPECIFIC PLASMID CLONES FOR USE IN NUCLEIC ACID HYBRIDIZATION REACTIONS

A variety of plasmid clones were obtained from the following individuals in order to examine expression of myogenic protein genes during BC3H1 myoblast cytodifferentiation. The generosity of these individuals is most gratefully acknowledged.

<table>
<thead>
<tr>
<th>Clone Description</th>
<th>Collaborator</th>
</tr>
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| VSM α-actin 5' flanking (intron1/exon 1) and 3'UT regions | Dr. Bonhong Min  
The Ohio State University & Kon Kuk University, Korea |
| SK α-actin 5' flanking (intron 1/exon 1) and 3'UT regions | Dr. Sandra Sharp  
Cal State Los Angeles |
| SK α-actin coding region, CA α-actin 3'UT region | Dr. Larry Kedes  
USC |
| VSM α-actin promoter region/CAT reporter gene fusion vectors | Dr. Michael Getz  
Mayo Clinic |
| Protein Id | Dr. Harold Weintraub  
Fred Hutchinson Cancer Research Center |
| Myogenin | Dr. Woodring Wright  
Southwest Medical Center  
University of Texas, Dallas |

Schematic maps presenting the essential features of these clones are presented in Appendix 1.

Near milligram quantities of the above clones were obtained by growing transformed *E.coli* and purifying the plasmid from the bacteria. All clones listed above were capable of transforming competent bacteria, which were then phenotypically selected by
resistance to either ampicillin or tetracycline. For clones carrying the Amp\textsuperscript{R} gene, the mv1190 strain of \textit{E.coli} was used as bacterial host. Competent cells of each strain were prepared by CaCl\textsubscript{2} shock, and were either transformed immediately or stored as glycerol stocks at \(-70^\circ\text{C}\) until needed (Sambrook et al. 1989). Following transformation as described by Sambrook \textit{et al}, cells were plated on Luria Broth (LB) agar plates containing ampicillin (50 \(\mu\text{g/ml}\)) or tetracycline (50 \(\mu\text{g/ml}\)) and incubated overnight at 37\(^\circ\text{C}\). Well isolated single colonies were picked and grown up as "minipreps" for 6-8 hours in 5 ml cultures of LB + antibiotics.

Plasmid DNA was isolated from miniprep cultures using the rapid LiCl-phenol plasmid preparation (Ausubel et al. 1991). Bacteria were pelleted by microcentrifugation and the cells lysed in TELT buffer containing 50 mM Tris Cl pH 8.0, 62.5 mM disodium EDTA, 2.5 M LiCl, 4% (v-v) Triton X-100. An equal volume of phenol (Gibco/BRL Life Technologies, Gaithersburg, MD): chloroform : isoamyl alcohol (25:24:1) was added, and the mixture vortexed vigorously for 15 seconds. After brief microcentrifugation, the aqueous phase was removed and the plasmid DNA and RNA precipitated by addition of two volumes absolute ethanol. The pellet was washed once in absolute ethanol, dried, and resuspended in either TE (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0) or double distilled water (ddH\textsubscript{2}O). This rapid plasmid preparation was also utilized to purify plasmid DNA on a larger scale, (50 and 250 ml cultures grown for 16 hours at 37\(^\circ\text{C}\)). For such preparative plasmid preparations, the RNA was digested by treatment with 0.1 \(\mu\text{g/\mu l}\) RNase for four hours at 37\(^\circ\text{C}\). The RNase subsequently
was removed by extraction with phenol followed by chloroform: isoamyl alcohol (24:1). Plasmid DNA was isolated by ethanol precipitation and was quantitated after dissolving small aliquots in water by measuring absorbancy at 260 nm. The identity of the amplified clone was confirmed by restriction endonuclease fragment mapping on agarose gels.

In some cases, existing clones were modified to generate new clones. The myogenin cDNA insert was removed from the plasmid expression vector, (pEMSVscribe α2), by digestion with the restriction endonuclease Eco R1. Linearized pEMSV was isolated on a low melting temperature NuSieve™ (FMC Bioproducts, Rockland, ME) agarose gel, and recircularized using T4 ligase (Boehringer Mannheim) via "in gel ligation" according to the manufacturer's instructions (FMC). This vector, lacking cDNA sequences, provided a convenient negative control for hybridization studies. In addition, it permitted the subcloning of the 688 bp SK α-actin 5' flanking region (intron 1/exon 1) obtained from Dr. Sandra Sharp out of the parent M13 vector into pEMSV using a Bam H1/Eco R1 unidirectional cloning approach and "in gel ligation". The identity of "pEMSV688SK" was confirmed by restriction mapping and was found to be functionally equivalent to the parent clone (clone 18-5, M.C-T. Hu et al. 1986) by comparing hybridization of pEMSV688 to a trial Northern blot with that obtained using clone 18-5 as a probe for an identical Northern blot (data not shown). Maps of pEMSV and of pEMSV688SK are provided in Appendix 1.
ANALYSIS OF GENE SPECIFIC mRNA EXPRESSION

Isolation of Total RNA

Cells at various stages of differentiation were harvested by scraping into 5 ml of ice cold PBS and collected by centrifugation at 500 x g for 10 minutes at room temperature. Cell pellets were frozen in liquid nitrogen and stored at -70° C. Total RNA was isolated from cells using the acid guanidinium thiocyanate phenol chloroform (AGPC) method of Chomczynski and Sacchi (Chomczynski and Sacchi 1987). Frozen cell pellets were disrupted by sonication in 5 ml of freshly prepared solution D containing 4M guanidine thiocyanate (Eastman Kodak), 25 mM sodium citrate, 0.5% sarcosyl, 0.1M β-mercaptoethanol. One tenth volume of 2M sodium acetate (pH 4.0) was added, followed by vigorous mixing with one volume of water saturated phenol (Gibco/BRL Life Technologies). One tenth volume of chloroform : isoamyl alcohol (24 : 1) was added and mixed by vortexing. After a 15 minute incubation on ice, the phases were separated by centrifugation at 10,000 x g at 4° C for 20 minutes, and the RNA contained in the upper, aqueous phase was collected by isopropanol precipitation. The next day the RNA was pelleted by centrifugation at 10,000 x g at 4° C for 20 minutes, and re-extracted in 2 ml of freshly prepared solution D followed by isopropanol precipitation. Resuspended RNA was further purified from residual sugars by ethanol precipitation, (one tenth volume 2.5 M sodium acetate pH 5.2, 2 volumes ice cold 95% ethanol), washed in 70%
ethanol, then dissolved in diethylpyrocarbonate-treated ddH₂O (DEPC ddH₂O). The RNA concentration and purity were determined by measuring absorbancy at 260 and 280 nm. Aqueous solutions of total RNA were stored at -70°C.

**Denaturing Gel Electrophoresis and Transfer of RNA Onto Solid Supports (Northern Blotting)**

Total cellular RNA was denatured in a mixture prepared according to the following recipe (Thomas 1980): to each 5 µl of RNA solution was added 17.8 µl of denaturing gel mix consisting of 10 µl formamide (Boehringer Mannheim, Indianapolis, IN), 3.6 µl freshly deionized 37% formaldehyde, 2 µl 10X buffer, (200 mM 3-[N-Morpholino] propane-sulfonic acid (MOPS, Sigma) pH 7.0, 50 mM sodium acetate, 5 mM EDTA) and 2.2 µl 30% glycerol. Samples were heated to 65°C for 10-15 minutes, then chilled on ice prior to loading into wells cast in 1.4% Seakem GTG™ agarose (FMC)/6.5% formaldehyde gels. Typically, 5 to 10 micrograms of total RNA was added per lane. The RNA samples were electrophoretically separated at 2-3 volts/cm constant voltage until the bromphenol blue tracking dye had traveled approximately seven centimeters. The position of 28S and 18S rRNAs was visually determined by ethidium bromide fluorescence. Prior to transfer, the RNA was denatured by soaking the gel in 50 mM NaOH/10mM NaCl for 30 minutes followed by neutralization in 0.1 M Tris HCl, pH 7.5 for 30 minutes. For capillary-diffusion transfer, the gel was further rinsed in 20X SSC (1X SSC = 15...
mM NaCl, 1.5 mM sodium citrate pH 7.0) for 30 minutes. Capillary diffusion blots were performed overnight using 20X SSC transfer buffer. For positive pressure transfer, no additional washes were required. RNA was transferred to Duralon (nylon membrane), or on occasion to Duralose (nylon reinforced nitrocellulose), (0.45 μM pore size, both from Stratagene, LaJolla, CA). Positive pressure transfer was accomplished using a Posiblot™ apparatus (Stratagene) set at 75-80 mm Hg for two hours. Following transfer, the RNA was covalently bound to the membrane by brief exposure to UV light in a Stratalinker™ UV crosslinking apparatus (Stratagene) and the blots stored in sealed plastic bags at 4°C until hybridization. The membranes were examined under UV light to determine uniformity of RNA transfer, and the gels were inspected for residual, untransferred RNA.

RNA-DNA Hybridization Methods

Hybridization with gene-specific probes was performed using cDNA plasmid clones or NuSieve™ gel-purified DNA fragments radiolabeled to high specific activity using α-[32 P]deoxyadenosine triphosphate (dATP) (≥ 3000 Ci/m mole, Amersham) in either nick-translation (specific activity=7.5 x 10⁷ - 1.25 x 10⁸ cpm/μg) or oligonucleotide random-primed reactions (specific activity ≥ 1.0 x 10⁹ cpm/μg) according to the manufacturer's instructions, (Nick Translation Kit and Multiprime DNA Labeling System, both from Amersham). Unincorporated nucleotides were separated from labeled
probe by spermine (10mM) precipitation using salmon sperm DNA as a carrier. Labeled probe plus the carrier DNA were pelleted by microcentrifugation at 4°C for 15 minutes, extracted for 1.5 hours with 200 μl of spermine extraction buffer, (75% ethanol, 0.3 M sodium acetate, 10 mM magnesium acetate), collected by microcentrifugation at 4°C for 10 minutes, and finally resuspended in 200 mL DEPC ddH₂O. Following a 3-4 hour prehybridization period at 42°C using 6X SSC / 1X BLOTTO (Johnson et al. 1984; Siegel and Bresnick 1986), the blots were incubated in fresh hybridization solution containing radiolabeled probe at a concentration of at least 300,000 cpm/ml (BLOTTO = 0.25% (w/v) nonfat instant dry milk. Prior to use, the SSC/BLOTTO solution was treated with 2.0% DEPC (v/v) to inactivate RNases). After a 36-48 hour hybridization period, non-specifically bound probe was removed by washing the blots four times for 15 minutes each with 2X SSC at 22°C, then twice with 0.2X SSC/0.2% SDS for 25 minutes each at 58 - 63°C. Hybridization was detected by exposure of the blot to Kodak XAR-5 x-ray film for up to 8 days using DuPont Cronex Lightning Plus intensifying screens. Exposures fell within the linear response range of the film. In some instances, Northern blot images were enhanced using a Tracor Northern TN8502 image analysis system and IPa85 software (Tracor Northern, Middleton, WI).
RESULTS

Strauch and Rubenstein have previously shown that the expression of vascular smooth muscle (VSM) α-actin is inducible in confluent BC3H1 cells upon withdrawal of serum from the culture medium. The induction of VSM α-actin was demonstrated at both the protein level, by peptide mapping (Strauch and Rubenstein 1984a), and at the mRNA level by Northern blot analysis (Strauch et al. 1986). Induction of VSM α-actin mRNA was accompanied by a corresponding decrease in the level of mRNA encoding for the nonmuscle β- and γ-isoactins. Such actin isoform switching is fully reversible in BC3H1 cells upon reseeding fully differentiated myocytes at subconfluent densities in serum-containing medium. However, high resolution electrophoretic analysis of tryptic digests of actin prepared from fully differentiated myocytes also revealed the presence of a novel minor peptide co-migrating with nonmuscle β- and γ-actin amino terminal tryptic peptides at pH 6.3 (Strauch et al. 1986). The electrophoretic mobility at pH 3.3 of this minor peptide corresponded to that expected for the amino terminal of a sarcomeric α-actin, or, alternatively, a non-acetylated biosynthetic intermediate of nonmuscle
β-actin (Rubenstein and Martin 1983). The purpose of the present study, therefore, was to identify this minor peptide and to determine if its expression is developmentally regulated during BC3H1 cytodifferentiation.

The six actins known to be expressed in mammalian species are remarkably homologous; the muscle actins are ≥ 98% conserved. Interestingly, the majority of the primary sequence differences between actins are clustered in the highly acidic amino terminal end of the molecule. The amino terminal tryptic peptides (Nts) of five actin isoforms, arranged to show sequence homology, are shown in Figure 1. Note that all amino terminal residues are acetylated and that each peptide contains several aspartic acid and glutamic acid residues, and at least one cysteine residue. Since the order of peptide migration on thin layer electrophoresis (TLE) depends on the charge to mass ratio of the peptide at a given pH, the highly acidic character of the actin amino termini permits their ready separation from the other peptides produced upon tryptic digestion of actin (Strauch and Rubenstein 1984b; Vandekerckhove and Weber 1978a; Vandekerckhove and Weber 1978b). In addition, the cysteine residue provides a convenient means for radiolabeling actin amino termini for electrophoretic analysis.

Autoradiographs of pH 6.3 thin layer electrophoretograms of tryptic digests of actin isolated by DNase I affinity chromatography from L-[35S]cysteine labeled BC3H1 cells at three different developmental stages are shown in Plate I. At pH 6.3 the acidic
FIGURE 1. Amino-terminal Tryptic Peptides (Nts) of Five Actin Isoforms Arranged to Show Sequence Homologies

The order of peptide migration during thin layer electrophoresis depends on the charge to mass ratio of the peptide at a given pH. Note that all amino termini are acetylated and that each peptide contains numerous aspartic acid and glutamic acid residues. At pH 6.3, all five peptides have a net negative (acidic) charge, although the vascular smooth muscle $\alpha$-actin Nt is the most acidic since it contains an extra cysteic acid residue following performic acid oxidation. The other four Nts will comigrate at pH 6.3. Downward pointing arrowheads (▼) indicate sites of thermolysin cleavage; upward pointing arrowheads (▲) indicate sites of cleavage by S. aureus V8 protease. Asterisks (*) and squares (□) indicate radioactive residues if actin is synthesized by cells in the presence of L-[35S]methionine or L-[35S]cysteine, respectively.
Skeletal Alpha-Actin Nt

Ac-Asp-Glu-Asp-Glu-Thr-Thr-Ala-Leu-Val-Cys-Asp-Asn-Gly-Ser-Gly-Leu-Val-Lys

Cardiac Alpha-Actin Nt

Ac-Asp-Asp-Glu-Glu-Thr-Thr-Ala-Leu-Val-Cys-Asp-Asn-Gly-Ser-Gly-Leu-Val-Lys

Vascular Smooth Muscle Alpha-Actin Nt

Ac-Glu-Glu-Glu-Asp-Ser-Thr-Ala-Leu-Val-Cys-Asp-Asn-Gly-Ser-Gly-Leu-Cys-Lys

Nonmuscle Beta-Actin Nt


Nonmuscle Gamma-Actin Nt

Ac-Glu-Glu- Glu- Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn- Gly-Ser-Gly-Met-Cys-Lys

FIGURE 1.
amino acid residues are all negatively charged, but the VSM α-actin Nt has the greatest charge to mass ratio by virtue of having two cysteic acid residues after performic acid oxidation. Consequently, the VSM α-actin Nt migrates farthest towards the anode, essentially co-migrating with the orange G marker. As seen in Plate I, panel A, subconfluent BC3H1 myoblasts in 10% serum-containing medium express low levels of VSM α-actin. Expression of VSM α-actin is up-regulated at confluence (Plate I, panel B), and reaches steady state level of expression after six days in hormone-supplemented serum-free N2 medium, (Plate I, panel C), at which point the cytoarchitectural changes which accompany biochemical differentiation are complete. This result therefore indicates that VSM α-actin synthesis is up-regulated at "confluence" in BC3H1 cells, prior to morphological differentiation, and that induction of α-actin synthesis is not absolutely dependent upon serum withdrawal.

Tryptic peptides derived from nonmuscle β- and γ-actin migrate behind the orange G marker, and appear in these autoradiographs as a well separated doublet. Note that distinct separation of the β- and γ-actin tryptic Nts is not routinely observed at pH 6.3 due to the similar charge to mass ratios at this pH, and that no determination has been made regarding which band corresponds to which peptide at this stage. Based on the theoretical charge to mass ratio of the isoactin Nt tryptic peptides at pH 6.3, a peptide derived from a sarcomeric actin (SK or CA), if present, would migrate "in the vicinity" of the peptides derived from the nonmuscle actins. Additionally, the band denoted by the star in Plate I corresponds to the carboxy terminal dipeptide
PLATE I. Autoradiographs Depicting pH 6.3 Thin Layer Electrophoretograms (TLEs) of L-[35S]Cysteine Labeled Actin Tryptic Peptides Prepared from BC3H1 Cells

Actin was processed from subconfluent myoblasts cultivated in DMEM 10% FBS (A), confluent myoblasts in DMEM 10% FBS (B), and well-differentiated myocytes exposed for six days to N2 serum-free medium (C). The upregulation of VSM α-actin during BC3H1 cytodifferentiation is evident when comparing all three panels (the position of the VSM Nt is indicated by the abbreviation SM). Tryptic peptides derived from nonmuscle β- and γ-actin, indicated by the abbreviation NM, migrate behind the orange G dye front which is indicated by the arrowhead. Note that distinct separation of β- and γ-actin Nts is not routinely observed at pH 6.3. Based on the charge to mass ratio of actin amino terminal tryptic peptides at pH 6.3, a peptide derived from a sarcomeric actin, if present in BC3H1 myocytes, would migrate in the vicinity of the nonmuscle actin Nts. The star indicates a Cys-Phe dipeptide derived from the actin carboxy terminal end; the letter O indicates the origin.
cysteine-phenylalanine. This dipeptide, which has been previously characterized (Strauch and Rubenstein 1984b; Vandekerckhove and Weber 1981), results from the tryptic cleavage of the peptide bond between lysine at position 372 and cysteine sulfonic acid and phenylalanine at positions 373-374 in all oxidized actin polypeptide chains. Peptides which remain closer to the origin represent less acidic actin tryptic peptides, and since these peptides are common to all actin isoforms they are not useful for actin isoform subtyping.

To determine if sarcomeric α-actin Nt peptides were present in tryptic digests of BC3H1 myocyte actin, all peptides comigrating with the nonmuscle actin Nts were eluted from the pH 6.3 TLE for secondary electrophoretic analysis at pH 3.3. At this more acidic pH, glutamic acid residues (pI = 3.22 at 25°C) are virtually uncharged, aspartic acid residues (pI = 2.77 at 25°C) are mostly charged, and cysteic acid residues are fully charged, (Vandekerckhove and Weber 1981; Cohn and Edsall 1943). However, since the nonmuscle and sarcomeric actin tryptic amino termini each have one cysteic acid residue, the relative rate of migration at pH 3.3 is determined by aspartic acid content. In the autoradiographs depicting pH 3.3 electrophoretograms in Plate II, panel A and Plate II, panel B, two bands are detected which correspond to myoblast β- and γ-actin eluted from the TLEs shown in Plate I. By virtue of its four aspartic acid residues, the β-actin Nt (peptide 1) migrates farther than the γ-actin Nt (peptide 2), which contains only one aspartic acid residue. Panels C-F of Plate II represent secondary electrophoretic analysis at pH 3.3 of all the actin amino terminal tryptic peptides co-migrating at pH 6.3
PLATE II. Autoradiographs Depicting pH 3.3 TLEs of Selected Peptides Eluted from pH 6.3 TLEs

All tryptic amino terminal peptides migrating in the vicinity of nonmuscle actin Nts prepared from L-[35S]cysteine labeled subconfluent myoblasts in DMEM, 10% FBS (A), confluent myoblasts in DMEM 10% FBS (B), and confluent myoblasts following cultivation in N2 serum-free medium for either 24 hours (C), 48 hours (D), 72 hours (E), or 144 hours (F) were eluted from pH 6.3 TLEs and analysed by secondary electrophoresis at pH 3.3. At pH 3.3, glutamic acid residues are virtually uncharged, aspartic acid residues are mostly charged, and cysteic acid residues are fully charged. In panels A and B only two bands are detected, corresponding to myoblast β- and γ-actin (peptides 1 and 2 respectively). In post-confluent myoblasts cultivated in N2 medium, however, a novel peptide (peptide 3) is resolved that migrates between β- and γ-actin Nts. Peptide 3 exhibits an electrophoretic mobility similar to that expected for a sarcomeric α-actin Nt peptide. An autoradiograph of L-[35S]methionine labeled actin tryptic peptides prepared from BC3H1 myocytes and separated at pH 3.3 is shown in panel G. Peptide 3 is not detected in this preparation. Note that only nonmuscle actin tryptic Nts contain methionine residues (refer to Figure 1). The letter O and the arrowhead denote the positions of the origin and the position of xylene cyanol FF marker dye, respectively.
with the nonmuscle actin tryptic Nts prepared from post-confluent BC3H1 myoblasts 24-144 hours after treatment with serum-free N2 medium. After 48 hours (Plate II, panel D), a novel band is resolved that migrates between the β- and γ-actin amino terminal tryptic peptides. A sarcomeric α-actin Nt, with three aspartic acid residues, would in fact be expected to migrate between the β- and γ-actin Nts. Significantly, the expression of this novel peptide (peptide 3) increases during BC3H1 cytodifferentiation, and reaches an apparent maximum after six days in N2 medium (plate II, panel F).

Rather than representing the amino terminal tryptic peptide of a sarcomeric α-actin, based on its electrophoretic mobility peptide 3 could alternatively be derived from an unprocessed nonmuscle β-actin biosynthetic intermediate previously described by Strauch and Rubenstein (Strauch et al. 1986). However, the β-actin biosynthetic intermediate is detected at all stages of BC3H1 differentiation, whereas the expression of the novel tryptic peptide resolved at pH 3.3 is clearly developmentally regulated in BC3H1 cells. In addition, this novel peptide is not detectable in actin tryptic digests prepared from fully differentiated BC3H1 myocytes labeled with L-[35S]methionine, (Plate II, panel G). Figure 1 shows that only nonmuscle actin tryptic amino terminal peptides contain methionine; sarcomeric actins have leucine residues at position 16 rather than methionine. Were the novel peptide in actuality derived from a β-actin biosynthetic intermediate, it should have been labeled by the radioactive methionine. These data therefore suggest that the novel amino
terminal tryptic peptide is derived from a sarcomeric α-actin and not an unprocessed nonmuscle isoactin.

In order to fully characterize this putative sarcomeric peptide and to determine whether it is derived from SK or CA α-actin, we pursued two different avenues of investigation. At the protein level, we isolated preparative amounts of the suspected sarcomeric α-actin tryptic amino terminal peptide and performed diagnostic secondary proteolytic digestions with thermolysin and Staphylococcus aureus V8 protease. At the RNA level, we employed isoform-specific probes to identify SK or CA α-actin mRNAs transcribed in fully mature BC3H1 myocytes.

The sites of cleavage of thermolysin and V8 protease are indicated in Figure 1. All actin amino termini contain an alanine-leucine thermolysin cleavage site between residues 7 and 8; in addition sarcomeric actin Nts contain an active second site between glycine at position 15 and leucine at position 16. Note that the VSM α-actin Nt also contains glycine-leucine at positions 15-16, but thermolysin does not cleave the VSM Nt at this site, probably because of the adjacent acidic cysteic acid residue (Vandekerckhove and Weber 1981). Proteolytic digestion of sarcomeric α-actin Nts at this second site results in the loss of the carboxy terminal tripeptide, yielding a peptide with a higher charge to mass ratio at pH 6.3 than the peptides produced after thermolysin digestion of the nonmuscle actin amino terminal tryptic peptides. The results of the secondary digestion of peptides 1, 2, and 3 with thermolysin are shown in Plate III. As the autoradiograph reveals, the peptide 3 derivative migrated
much farther than the peptide 1 or 2 derivatives (compare Plate III, panel B with Plate III, panels A and C). This result is consistent with the suggestion that peptide 3 does represent the amino terminal tryptic peptide of a sarcomeric α-actin.

Final characterization of peptide 3 was obtained by secondary digestions with V8 protease. When used in a bicarbonate buffer, V8 protease preferentially hydrolyzes peptide bonds on the carboxy-terminal side of glutamic acid residues. Sarcomeric α-actin tryptic Nts and nonmuscle γ-actin tryptic Nts are each cut by V8 protease on the carboxy side of the glutamic acid residue at position 4, resulting in peptides with identical charge to mass ratios (Vandekerckhove and Weber 1981). Nonmuscle β-actin tryptic Nts lack glutamic acid residues and are therefore largely resistant to V8 protease digestion. Thus sarcomeric α-actin and nonmuscle β-actin amino termini tryptic peptides can be distinguished based on the electrophoretic behavior of the V8 protease digestion products. We expect that the undigested β-actin tryptic peptide will migrate faster at pH 6.3 than either the sarcomeric α-actin or γ-actin V8 protease peptides, since it contains 5 charged amino acid residues. As the autoradiograph depicted in Plate IV reveals, peptides 2 and 3 are in fact cleaved by V8 protease to yield peptides with identical electrophoretic properties at pH 6.3. Peptide 1 was not cut by V8 protease, and migrates substantially farther than the digestion products derived from peptides 2 or 3. Note that the doublets are the consequence of a partial deamidation of the asparagine residue at position 12 under the alkali conditions used during the V8 protease digestions; this results in an additional
PLATE III. Autoradiograph of a pH 6.3 TLE Separating the Products Obtained by Thermolysin Digestion of the Following Actin Tryptic Nts Identified in Plate II: Peptide 1 (A), Peptide 3 (B), Peptide 2 (C).

As shown in Figure 1, thermolysin cleavage of β- and γ-actin tryptic Nts yields a peptide containing 11 residues. In contrast, a sarcomeric α-actin tryptic Nt would be cleaved by thermolysin at two positions to yield a peptide with 8 amino acids and having a greater charge to mass ratio than either of the nonmuscle actin tryptic Nts. This result indicates that the peptide 2 might correspond to an authentic sarcomeric α-actin tryptic Nt. The origin and the orange G dye front are denoted by the letter O and an arrowhead, respectively.
PLATE IV. Autoradiograph of a pH 6.3 Electrophoretogram Depicting
the Products Obtained by S. aureus V8 Protease Digestion
of the Following Actin Tryptic Nts Identified in Plate II:
Peptide 1 (A), Peptide 3 (B), Peptide 2 (C).

Final characterization of peptides 1, 2, and 3 is obtained by
digestion with S. aureus V8 protease. Sarcomeric α-actin and
nonmuscle γ-actin tryptic Nts are each cleaved by V8 protease to
produce peptides having an identical charge to mass ratio at pH 6.3
(refer to Figure 1). The β-actin tryptic Nt is not cleaved by V8
protease. Consequently, the β-actin Nt migrates faster at pH 6.3 than
the comigrating, more basic sarcomeric α-actin and γ-actin V8
protease digestion products. Under the alkali conditions used in V8
protease digestion reactions, the asparagine residue at position 12 in
each actin Nt is partially deamidated to aspartic acid, resulting in a
more acidic peptide. This partial deamidation reaction results in a
doublet; the more acidic form of each peptide is marked by an
asterisk.
aspartic acid residue and thereby increases the charge to mass ratio of the deamidated peptide compared to its unmodified counterpart (Vandekerckhove and Weber 1981). These data clearly indicate that the novel actin amino terminal tryptic peptide (peptide 3) is derived from a sarcomeric α-actin expressed in fully-differentiated BC3H1 myocytes.

Vandekerckhove *et al* (1986) were able to distinguish SK α-actin from CA α-actin by employing partial acid hydrolysis of the amino terminal tryptic peptides. We were unable to obtain sufficient quantities of the sarcomeric α-actin Nt to perform isoactin subtyping at the peptide level; as an alternative approach we employed actin isoform gene-specific cDNA probes to identify the presence of SK or CA α-actin mRNAs on Northern blots. An autoradiograph of a Northern blot of total RNA isolated from BC3H1 myoblasts (lane 1) and 6d myocytes (lane 2) and hybridized to an actin coding region probe is shown in Plate V, panel A. Myoblasts express predominantly nonmuscle β- and γ-actin; the mRNA transcripts encoding these isoactins are approximately 2100 nucleotides in length. As seen previously in Plate I, myoblasts do express low levels of VSM α-actin; the mRNA transcripts encoding muscle actins are approximately 1500 nucleotides in length. The induction of muscle actin mRNA and the down-regulation of nonmuscle actin transcription during BC3H1 differentiation previously described by Strauch *et al* (1986) is evident in Plate V, panel A. The actin coding region probe hybridizes with mRNA for all isoactins by virtue of the high degree of homology between the various isoactins; in contrast, the actin gene-specific
PLATE V. Autoradiographs of Northern Blots Showing Total RNA from BC3H1 Cells and Mouse Skeletal and Cardiac Muscle Probed with Actin Isoform-Specific Probes

Total RNA was isolated from BC3H1 myoblasts (lane 1, panel A) and 6 day myocytes in N2 medium (lane 2, panels A-C), and from mouse skeletal muscle (lane 1, panel B) and cardiac muscle (lane 1, panel C). The blot shown in panel A was probed with pHMaA-1, a SK actin coding region probe which hybridizes with all actin transcripts. While 2100 nucleotide transcripts for β- and γ-actin were the major mRNA species detected in myoblasts, 1500 nucleotide α-actin mRNAs predominated in differentiated myocytes. The position of RNA size markers is shown on the left of panel A. The blot shown in panel B was probed with pJ3', a plasmid containing a cDNA insert corresponding to the 3'UT region of SK α-actin, while the blot depicted in panel C was probed with pHMcA-3'UT-DB, a cDNA probe specific for the 3'UT region of CA α-actin. This result indicates that mature BC3H1 myocytes express SK α-actin mRNA but not CA α-actin mRNA.
cDNA clones are homologous to the highly divergent, isoform-specific 3' untranslated regions of the corresponding messenger RNA transcripts. The autoradiograph in Plate V, panel B reveals that SK α-actin mRNA is present in BC3H1 myocytes, whereas there is no evidence of CA α-actin mRNA in the same 6d myocyte population, (Plate V, panel C). Based on these data, we conclude that SK α-actin is expressed during BC3H1 differentiation, and that its expression occurs after the observed up-regulation of VSM α-actin.
DISCUSSION

The peptide mapping and Northern blot analyses of actin isoform expression during BC3H1 cytodifferentiation presented here demonstrate that vascular smooth muscle and skeletal muscle α-actins are co-expressed in BC3H1 myocytes. The observation that VSM α-actin expression occurs in confluent myoblasts cultivated in serum-containing medium, while expression of SK α-actin expression occurs relatively late in the developmental program after morphological differentiation has already begun, suggests that the vascular smooth muscle and skeletal muscle α-actin genes are independently regulated during myogenesis.

The DNA sequences and intron/exon structures of the isoactin genes are quite similar, leading to the speculation that co-expression of the sarcomeric α-actin genes during skeletal myogenesis may be coordinately controlled by similar transcriptional regulatory signals (Hayward and Schwartz 1986). Indeed, a comparison of the 5' flanking sequences of the CA, SK, and VSM α-actin genes reveals some homologies, particularly with regard to the presence of multiple sequence motifs having the consensus sequence CC(A/T)₆GG.
(Buckingham 1985; Carroll et al. 1986; Carroll et al. 1988; Wade and Kedes 1989; Min et al. 1990). Furthermore, the observation that the myogenic "master regulatory gene" myoD will simultaneously activate a variety of muscle-specific genes upon transfection into nonmuscle cell types suggests that all developmentally regulated muscle-specific genes may in fact be responsive to similar regulatory signals. However, support for the concept of independent regulation of co-expressed α-actins is obtained from Hayward and Schwartz (Hayward et al. 1988), who examined the expression of actin genes during chick myogenesis. In primary cultures of embryonic myoblasts, CA α-actin expression was induced upon withdrawal from the cell cycle and remained elevated after myoblast fusion. Conversely, SK α-actin was induced in a fusion-dependent process and levels gradually declined post-fusion. Taken together, these experimental results suggest that although there may be universal signals which permit expression of muscle-specific genes during myogenesis, the timing and fine-tuning of muscle-specific gene expression may depend on other, as yet uncharacterized, regulatory signals and/or trans-acting factors. Experiments designed to investigate the regulatory mechanisms governing α-actin expression in BC3H1 cells are described in the second and third chapters of this dissertation.

The experiments described in this chapter document the developmentally regulated co-expression of VSM and SK α-actin isoforms in BC3H1 cells, and they therefore force us to critically re-evaluate earlier suggestions that BC3H1 cells are of smooth muscle origin. Isolated from a chemically induced mouse brain neoplasm,
BC3H1 cells were thought to be derived from smooth muscle cells on the basis of morphological, biochemical, and physiological analyses. This classification, first proposed by Schubert (Schubert et al. 1974), was supported by the observation that BC3H1 cytodifferentiation led to the accumulation of VSM α-actin (Strauch and Rubenstein 1984a). However, in addition to our finding that BC3H1 cells express SK α-actin, Taubman et al (1989) have shown that differentiated BC3H1 cells induce expression of troponin T and also express sarcomeric muscle-specific isoforms of proteins such as myosin heavy chain, myosin light chain 2 and 3, and α-tropomyosin.

It is not unreasonable that smooth muscle-like cells should be derived from a brain neoplasm, either as components of nascent arterioles or as stromal myofibroblasts. Indeed, Skalli et al (1986) have shown that myofibroblasts not only synthesize vascular α-actin but may also be associated with neoplastic growths. They hypothesize that this association might be promoted by tumor secretion of factors which stimulate stromal fibroblast growth and differentiation into contractile myofibroblasts. In light of the ability of the DNA demethylating agent 5-azacytidine to stably convert the phenotype of undifferentiated mesenchymal fibroblasts into differentiated myocytes, chondrocytes, and adipocytes, it is tempting to speculate that the BC3H1 cell line may have arisen from tumor-associated myofibroblasts which attained the ability to express SK α-actin as a result of mutation or undermethylation.
Another possibility is that BC3H1 cells represent benign transformations of either smooth muscle (leiomyoma) or skeletal muscle (rhabdomyoma). Skalli and Schurch recently reported the results of an extensive morphological, biochemical, and immunohistochemical analysis of the cytoskeletal phenotypes of fifteen assorted leiomyosarcomas and leiomyomas and fifteen rhabdomyosarcomas (Schurch et al. 1987; Skalli et al. 1988). Among the smooth muscle cell tumors, VSM \( \alpha \)-actin expression was highest in well-differentiated leiomyomas. Although SK \( \alpha \)-actin expression was detected in two malignant tumors, in none of the tumors was co-expression of VSM and SK \( \alpha \)-actin observed. Among the tumors of skeletal muscle origin, co-expression of VSM and SK \( \alpha \)-actin was observed in one case. Interestingly, this particular rhabdomyosarcoma expressed more VSM than SK \( \alpha \)-actin, and also displayed a spindle shaped cellular morphology devoid of sarcomeres and contained bundles of cytoplasmic thick and thin filaments in association with dense body structures. As described, this particular rhabdomyosarcoma shares many phenotypic traits with BC3H1 cells, and in view of these similarities it is tempting to speculate that BC3H1 cells may be derived from a skeletal muscle cell tumor. Further support for this argument is derived from the observation by Woodcock-Mitchell et al. (1988) and by Sawtell and Lessard (1989) that skeletal myoblasts express low levels of VSM \( \alpha \)-actin early in development. Thus, as Taubman et al. have suggested, BC3H1 cells may represent a skeletal muscle cell line of mesodermal origin that is
non-fusing and therefore defective for commitment to terminal differentiation.

In their characterization of an anti-sarcomeric α-actin monoclonal antibody, Skalli et al report that although the antibody did not react with VSM α-actin, on occasion a faint positive reaction was obtained in vascular smooth muscle cells during immunohistochemical staining of small vessels (Skalli et al. 1986). Interestingly, immunohistochemical studies of arterial smooth muscle cells have revealed at least three different populations of smooth muscle cells in the tunica media which can be distinguished by their pattern of cytoskeletal protein isoform expression (Skalli et al. 1986), and Fatigati and Murphy (Fatigati and Murphy 1984) have also demonstrated myofibrillar protein heterogeneity in vascular smooth muscle isolated from a variety of tissues. This vascular structural heterogeneity is apparently paralleled in vivo at the level of functional response to biological and environmental modifiers; the process of arteriosclerosis tends to involve smooth muscle cells in larger elastic-type arteries, whereas hypertension tends to affect the smooth muscle cells found in the muscular-type, smaller caliber "resistance vessels". Finally, although authentic vascular smooth muscle tissues and cells from adult rabbit and mouse do not express either SK α-actin protein or mRNA, the observation that leiomyosarcomas can express SK α-actin indicates that the SK α-actin gene can be activated in smooth muscle cells. Therefore, unless some unique protein marker is discovered that can be used to unequivocally determine their tissue ancestry, the origin of BC3H1 cells will continue to be unknown and
debatable. What the current study does help to establish, however, is that BC3H1 myogenic cells are a useful system for studying the developmental regulation of $\alpha$-actin isoform co-expression during myogenesis.
During muscle differentiation there is a profound shift in contractile protein gene expression as the nonmuscle and "embryonic muscle" isoforms that characterize immature myoblasts are replaced with the "muscle-specific" isoforms that comprise mature muscle fibers. The transition from an embryonic contractile protein isoform "profile" to that characteristic of an adult is apparently not, in the strictest sense, temporally coordinated, since the embryonic isoform of one contractile protein may persist during development for a longer time than the embryonic isoform of another muscle-specific protein. In other words, a developmental "switch" which uniformly "turns off" the expression of embryonic isoforms and which simultaneously "turns on" the expression of adult isoforms apparently does not exist. However, the absence of such strict temporal coordination should not be taken as evidence that isoform switching is not regulated. Indeed, the regulation of muscle-specific gene expression appears to be quite complicated and in many respects extremely subtle. In general,
however, muscle-specific gene transcription during myogenesis appears to be largely regulated at the level of gene transcription (Shani et al. 1981; Strohman and Wolf 1983).

It is well established that during vascular smooth muscle (VSM) development in vivo, nonmuscle β- and γ-actin isoforms are largely replaced with VSM α-actin. In developing rat aortic smooth muscle cells, actin isoform switching is completed by six weeks (Kocher and Gabbiani 1986); in the human, aortic smooth muscle contains "adult" levels of VSM α-actin by 24 weeks (Glukhova et al. 1990). Northern blot analysis of total RNA isolated from vascular smooth muscle in rats at different developmental stages indicates that α-actin RNA levels generally parallel α-actin protein levels in both quiescent and proliferating vascular smooth muscle (Vandekerckhove et al. 1986). This observation suggests that actin expression in developing rat smooth muscle is regulated primarily at the level of gene transcription. Evidence exists, however, for other means of regulating actin expression. For example, although α-actin is the dominant actin mRNA species expressed in adult rat VSM, β-actin is the dominant actin expressed in in vitro translation assays of mRNA isolated from adult rat VSM (Barja et al. 1986; Kocher and Gabbiani 1986). This result implies that β-actin mRNAs are translated more efficiently than α-actin mRNAs in vitro, a conclusion which is supported by a report documenting preferential in vitro translation of β-actin mRNA in RNA prepared from myogenic BC3H1 cells (Strauch et al. 1986). In addition, L-[35S]methionine pulse labeling studies show that β-actin is preferentially synthesized in adult rat VSM (Barja et al. 1986). If
β-actin is the major actin synthesized in adult muscle, as these studies suggest, then in order for VSM α-actin protein to accumulate to levels that exceed those of β-actin during muscle development we must hypothesize that either β-actin has an extremely short half-life in muscle cells or that α-actin has a prolonged half-life (or a combination of these two possibilities). In this regard, the acetylated biosynthetic intermediate of VSM α-actin has been found to have a longer half-life than other actins in BC3H1 cells (Strauch and Rubenstein 1984b).

Although there is, therefore, some evidence for post-transcriptional control of β-actin expression during vascular smooth muscle development in vivo, it appears that α-actin gene expression is regulated primarily at the level of gene transcription. However, there is evidence which suggests that a variety of factors exist which may influence the transcriptional control of α-actin gene expression in smooth muscle cells. Immunohistochemical studies have revealed that the tunica media of rat and human aorta contains three distinct populations of smooth muscle cells that can be distinguished based on their VSM α-actin and intermediate filament staining properties (Skalli et al. 1986). A similar observation was made by Glukhova et al. (1988) who describe a subendothelial population of smooth muscle cells that express low levels of adult phenotypic markers. In addition, Gabbiani et al. (1987) and Fatigati and Murphy (1984) observed that the proportion of actin isoforms expressed in smooth muscle cells isolated from various organs differs. Taken together, these observations suggest that smooth muscle cell development and actin isoform expression may be influenced by environmental factors.
Gurdon's observation that a small cluster of mesodermal cells is required before α-actin transcription is induced during Xenopus development (the so-called "community effect") supports the idea that the microenvironment can influence α-actin transcriptional regulation (Gurdon 1989). Additionally, the α-actin content of medial smooth muscle cells obtained from a hypotensive section of rat abdominal aorta was reduced when compared to normotensive and hypertensive controls (Kocher and Gabbiani 1987). These observations suggest that physiological signals also mediate α-actin expression in the fully developed animal.

Much of the recent work investigating actin regulation in vascular smooth muscle cells has been performed using cultured cell models. Subconfluent primary cultures of vascular smooth muscle cells undergo phenotypic modulation in vitro, expressing increased levels of nonmuscle β- and γ-actin and reduced amounts of α-actin. Alpha-actin synthesis in primary cultures is up-regulated at confluence by a process which apparently does not require cell cycle withdrawal (Blank et al. 1988; Kocher and Gabbiani 1987; Owens et al. 1986; Strauch et al. 1991). Interestingly, α-actin gene transcription is not inducible in VSM primary cultures; α-actin mRNA levels remain elevated even in proliferating smooth muscle myoblasts, comprising nearly 82% of the total actin mRNA (Kocher and Gabbiani 1987). However, α-actin represents only 17.5% of the total actin in these cells, suggesting that a translational or post-translational control mechanism is employed to regulate α-actin accumulation in primary cultures of VSM cells. With continued passage, however, the levels of
α-actin mRNA and protein in primary cultures become more and more similar. Kocher and Gabbiani found that after primary cultures had reached passage 5 the α-actin mRNA level more nearly approximated the relative α-actin protein content. At this stage, α-actin expression remains low, accounting for only 5-6% of the total cellular actin (Gabbiani et al. 1984; Kocher and Gabbiani 1987, Campbell et al. 1989), and the α-actin mRNA level is correspondingly low. As we have seen, this correlation between α-actin mRNA abundance and protein level is also observed in vivo. α-actin expression in passage 5 cells is not inducible, increasing only slightly to 12% upon attainment of confluence. Additional evidence for post-transcriptional control of α-actin expression in cultured VSM was presented by Corjay et al. (1990), who demonstrated that addition of platelet derived growth factor (PDGF) to quiescent confluent cultures results in dramatically reduced amounts of VSM α-actin mRNA and protein, although gene transcription levels are unchanged. Thus, while the principle mode of regulating α-actin expression in vascular smooth muscle in vivo appears to be at the level of gene transcription, primary culture conditions may exert unique transcriptional and translational controls on VSM α-actin accumulation. It remains to be seen if these controls are also active in vivo during embryogenesis and during the phenotypic modulation of VSM which occurs during smooth muscle proliferation.

Cardiomyocyte differentiation during embryogenesis is characterized by the sequential activation of two sarcomeric α-actin genes. Interestingly, however, a low level of VSM α-actin expression actually marks the onset of cardiogenesis (Ruzicka and Schwartz
CA α-actin mRNA is detected in developing heart muscle shortly after the appearance of VSM α-actin. With the onset of intra- and extra-embryonic circulation, VSM α-actin is down-regulated and SK α-actin is co-expressed with CA α-actin. Heart muscle from adult pig and cow ventricle was found to contain slightly over 80% CA α-actin and just under 20% SK α-actin (Vandekerckhove et al. 1986). This value correlates reasonably well with the relative amounts of CA and SK α-actin mRNAs in adult mouse heart, and suggests that transcriptional controls play a significant role in regulating α-actin expression during cardiac development. The significance of continued expression of two muscle-specific actin isoforms in adult cardiac muscle is not well understood.

Cardiomyocytes can be cultured in vitro, but unlike cultured VSM cells, cultured cardiomyocytes maintain an isoactin profile that is characteristic of mature cardiac muscle in vivo. However, the pattern of actin expression can be altered in response to the addition of growth factors or drugs. In vitro induction of α-1-adrenoreceptor-mediated hypertrophy results in a preferential increase in the level of SK α-actin transcripts (Bishopric et al. 1987). This observation, in conjunction with the determination that hypertrophic human heart muscle contains increased levels of SK α-actin mRNA (Gunning et al. 1983), suggests that induction or up-regulation of the fetal actin isogene transcription is a common muscle cell response to injury or stress, even in the absence of cell proliferation (Izumo et al. 1988; Simpson et al. 1989; Parker et al. 1990).
As with cardiogenesis, the onset of rat skeletal muscle differentiation \textit{in vivo} is marked by the expression of VSM $\alpha$-actin. VSM $\alpha$-actin protein is detected via \textit{in situ} hybridization in somitic myotomal cells shortly after it is detected in developing cardiomyocytes on gestational day 10, and the corresponding mRNA is detected in total RNA derived from developing muscle (Sawtell and Lessard 1989; Woodcock-Mitchell et al. 1988). Subsequently, VSM $\alpha$-actin expression is down-regulated and CA $\alpha$-actin is expressed in skeletal myoblasts. Shortly thereafter, SK $\alpha$-actin expression is up-regulated. Hybridization experiments have shown that by chicken stage 43, SK $\alpha$-actin mRNA accounts for approximately 90\% of the total actin transcripts (Hayward and Schwartz 1986). While there is no direct evidence that $\alpha$-actin expression in developing skeletal muscle is regulated at the level of gene transcription, the strong correlation between the levels of SK $\alpha$-actin protein and mRNA is suggestive of predominantly transcriptional control. In addition, a recent \textit{in situ} hybridization and immunohistochemical study demonstrated that SK $\alpha$-actin protein co-localizes with SK $\alpha$-actin mRNA. Furthermore, there is no observable delay between the detection of $\alpha$-actin mRNA and $\alpha$-actin protein, suggesting that translational controls are minimal (Lyons et al. 1991).

Primary culture of dissociated skeletal myoblasts is possible, and permits the study of skeletal myogenesis \textit{in vitro}. Cultured skeletal myoblasts proliferate, withdraw from the cell cycle at confluence, fuse to form myotubes, and express muscle-specific proteins upon biochemical differentiation. Significantly, experiments with myoblasts...
grown in calcium-deficient medium have demonstrated that initiation of myogenic differentiation \textit{in vitro} does not require cell cycle withdrawal or myoblast fusion \cite{Endo1987}. This result is in agreement with the observation that vascular smooth muscle cells do not need to withdraw from the cell cycle in order to express VSM $\alpha$-actin. However, the regulation of $\alpha$-actin expression in primary cultures of skeletal myoblasts may differ significantly from the \textit{in vivo} situation. Cultured embryonic chicken skeletal myoblasts have been shown to express CA $\alpha$-actin within twenty four hours after plating and $\beta$-actin mRNA levels also increase during the phase of myoblast proliferation. SK $\alpha$-actin mRNA is expressed only after myoblast fusion, and $\alpha$-actin mRNA levels steadily decline post-fusion, but in contrast to embryonic development, CA $\alpha$-actin remains the major actin isotype expressed even after fusion. Thus, the sequential appearance of $\alpha$-actins in cultured myoblasts parallels that observed during embryogenesis, but the continued high level of CA $\alpha$-actin expression suggests that primary cultures of skeletal myoblasts lack essential regulatory signals or factors for complete biochemical differentiation. A detailed \textit{in situ} hybridization and immunocytochemical study of primary cultures of skeletal myoblasts confirmed the basic pattern of $\beta$-actin down-regulation and asynchronous $\alpha$-actin induction and co-expression during differentiation \cite{Lawrence1989}. Interestingly, however, no CA $\alpha$-actin protein was detected in CA $\alpha$-actin mRNA positive myogenic cells until after fusion. These data imply that CA $\alpha$-actin expression is regulated in pre-fusion myoblasts by a post-transcriptional mechanism.
Although the single cell resolution made possible by the primary culture system employed by Lawrence and Singer surpasses the resolution of the developmental immunohistochemical study undertaken by Lyons, it may be that the observed delay in CA α-actin mRNA translation is a consequence of placing the myoblasts in culture. Recall that primary cultures of vascular smooth muscle also display evidence of post-transcriptional regulation in early passages.

Unlike smooth muscle and cardiac muscle, a number of immortal skeletal muscle cell lines exist which permit experimental investigation of myogenesis in vitro. Work with these model systems has been instrumental in determining that expression of muscle-specific genes during skeletal myogenesis in vitro may also be regulated by post-transcriptional processes such as alternative splicing, and by translational processes such as restricting message availability (Endo and Nadal-Ginard 1987). Significantly, the argument for translational control of several muscle-specific genes is based on observations in myoblasts experimentally blocked from fusing, perhaps making it unlikely that this hypothetical control mechanism functions during normal muscle development in vivo. However, these results may be important in understanding the regulation of α-actin expression in BC3H1 myogenic cells, which are fusion incompetent.

In regard to BC3H1 myogenic cell differentiation, it is interesting to note that preliminary evidence from our laboratory shows that multiple VSM α-actin mRNAs are expressed in BC3H1 cells at low levels, and that the size distribution of these mRNAs changes during differentiation (B. Min, Ph.D. dissertation). The significance of
these multiple VSM α-actin transcripts in BC3H1 cells is not yet clear, but multiple VSM α-actin mRNAs have also been detected in avian aortic smooth muscle cells (Carroll et al. 1986). At least four VSM α-actin mRNA transcripts have been identified in avian aortic smooth muscle cells. The four mRNAs range in size from 1370 to 2700 nucleotides and differ in the length of their 3' untranslated (3' UT) regions. It is believed that the different transcripts arise via the use of alternative polyadenylation sites during transcription. Whether or how these four different actin transcripts are involved in regulating VSM α-actin transcription is unknown, but studies of the transcriptional regulation of transfected SK α-actin/nonmuscle β-actin chimeric genes in BC3H1 cells support the hypothesis that the 3' UT portions of actin genes play an important role in the developmental regulation of actin expression (Sharp et al. 1989). That the 3' UT of VSM α-actin is both species- and isoform-specific, coupled with the fact that mRNA molecules of varying sizes have not been detected for the nonmuscle actins or for SK and CA α-actin, suggests that the expression of VSM α-actin during myogenesis may indeed be regulated in a manner different from those controlling expression of all other actins. Thus, although actin gene expression during myogenesis appears to be regulated at the level of gene transcription, other control mechanisms may function in conjunction with transcriptional regulatory mechanisms to "fine tune" actin biosynthesis in muscle cells.

Knowledge of the transcriptional status of a gene carries with it certain implications regarding the chromatin structure in the vicinity of the gene. For example, actively transcribed genes have been shown
to possess a conformation that is susceptible to digestion with 
deoxyribonuclease I (DNase I), whereas "silent" genes are not 
susceptible to DNase I digestion (Weintraub and Groudine 1976).
Recent work has shown that DNA methylation affects the formation of 
transcriptionally active chromatin, as assayed by DNase I sensitivity 
and restriction endonuclease susceptibility (Keshet et al. 1986; 
Pourcel et al. 1990; Selker 1990). These findings indicate that 
cytosine methylation may provide one mechanism by which cells 
regulate the developmental expression of tissue-specific genes (Cedar 
and Razin 1990). Yisraeli found that a methylated α-actin gene was 
not expressed when introduced into a fibroblast cell, but when the 
same construct was transfected into a myoblast cell line it was 
demethylated and expressed (Yisraeli et al. 1986; Paroush et al. 1990).
It has been observed that all housekeeping genes contain a GC-rich 
sequence (referred to as a CpG island) at their 5′ end. This sequence 
typically contains within it a smaller sequence motif that serves as the 
binding site for the transcription factor Sp1. GC-rich islands in active 
housekeeping genes are unmethylated, but housekeeping genes which 
are inactive, (such as those found on the Lyonized X chromosome in 
females), are associated with heavily methylated CpG islands (Lewin 
1990). In addition, in certain cell lines which no longer express 
tissue-specific proteins characteristic of the tissue of origin, the 
inactive genes are associated with heavily methylated CpG islands 
(Antequera et al. 1990). Apparently, methylation permits a methyl-
CpG binding protein to bind to the CpG island thereby preventing Sp1 
binding and the subsequent initiation of transcription (Boyes and Bird
This methyl-CpG binding protein may also contribute to the formation of a condensed, transcriptionally silent chromatin structure (Adams 1990). Recent work has also shown that cis-acting sites may be equally important in regulating the timing of demethylation during transcriptional activation of tissue-specific genes (Paroush 1990). These data, taken together with the results indicating that \( \alpha \)-actin expression during muscle development is regulated at the level of gene transcription, make it reasonable to inquire if modification of gene methylation status is the mechanism which governs \( \alpha \)-actin transcriptional regulation.

One strategy for investigating the role of methylation in gene expression is to perturb the normal genomic methylation patterns and to observe any phenotypic or biochemical consequences which might ensue. The nucleoside analog 5-azacytidine (5-azaC) can be used to reduce the level of DNA methylation \textit{in vitro}. In the mammalian genome, up to seven percent of all cytosine residues are methylated at the carbon 5 position (Lewin 1990). The majority of these methylated cytosines are found either in the mini-palindromic sequence CG, or within clusters of CG doublets referred to above as CpG islands. When cells are cultured in medium containing micromolar concentrations of 5-azaC, the analog becomes converted within the cell to 5-azadeoxycytidine and is randomly incorporated into the genome during replication. Once incorporated, it resists methylation by the cellular maintenance methylase and upon the next round of replication the progeny cell becomes stably demethylated at that locus. The number of loci converted is proportional to the concentration of
5-azaC; Jones and Taylor (1980) estimated that cells exposed to 2 μM 5-azaC for 24 hours undergo a 62% reduction in the level of cytosine methylation, while 10 μM results in an 80-85% reduction.

Taylor and Jones also discovered that treatment with 5-azaC resulted in stable phenotypic conversion of mouse C3H 10T1/2 embryonic fibroblasts into one of three mesodermal cell lineages at the following frequencies: myoblast (30%), chondroblast (2%), and adipoblast (8%) (Taylor and Jones 1979). Konieczny and Emerson (1984) proposed the existence of regulatory genes controlling cellular determination based on the ability of 5-azaC to phenotypically convert embryonic fibroblasts to myoblast, chondrocyte, and adipocyte lineages, and shortly thereafter the first myogenic regulatory factor, myoD, was identified (Lassar et al. 1986; Davis et al. 1987). MyoD, which stands for myoblast determination gene, was originally cloned by subtraction hybridization out of a cDNA library prepared from 5-azaC myoblasts. When a myoD expression vector is transfected into a variety of nonmuscle cell types, muscle-specific genes are activated in the nonmuscle cells (Weintraub et al. 1989). This suggests that myoD functions as a master regulatory gene during skeletal muscle development, promoting transcription of muscle-specific genes during differentiation.

A nuclear phosphoprotein found only in skeletal muscle cells, myoD has been shown to function as a positive activator of muscle creatine kinase (MCK) transcription by binding to 'E box' elements within the MCK enhancer region (E boxes have a consensus sequence of CANNTG, Murre et al. 1989 a,b). A 60-70 amino acid domain on the
amino terminal half of the myoD protein is highly homologous to a similar domain found in the protooncogene c-myc, and is sufficient for muscle-specific gene activation. This domain consists of a stretch of 10-20 basic amino acids required for DNA binding specificity adjacent to an amphipathic region of 40-50 amino acids which assumes a helix-loop-helix (HLH) secondary structure and which mediates dimerization of myoD with itself and with other HLH trans-acting factors. Dimerization is an important mechanism by which myoD binding to DNA is regulated (Weintraub et al. 1991; Lassar et al. 1991).

Since the discovery of myoD, a least four other muscle regulatory factors have been identified by their ability to induce muscle-specific gene expression in nonmuscle cells: myogenin, myf 5, mrf 4/herculin/myf 6, and myd (Braun et al. 1989; Konieczny and Emerson 1984; Pinney et al. 1988; Wright et al. 1989). With the exception of myd, these genes have all been sequenced and found to contain basic-HLH domains which are required for activity of the gene products. Like myoD, each is found only in skeletal muscle cells. Myogenin is perhaps the most universal of all the muscle regulatory factors, including myoD, since it is the only regulatory factor which has been detected in every skeletal muscle cell line. This and other observations suggest that the regulation of transcriptional activation of muscle-specific genes during myogenesis is not a simple matter of activating only myoD. Rather, other muscle regulatory factors are apparently also involved, and it is not unlikely that these factors form a regulatory cascade of gene activation in which not only muscle-specific genes and other myogenic factors are turned on but also one in which
muscle inhibitory factors are turned off in response to as yet unidentified developmental signals (Blau 1988, Jones 1990). One such inhibitory factor recently cloned and sequenced is protein Id, (for inhibition of DNA binding, or inhibition of differentiation) (Benezra et al. 1990). Id is found in all cell types analyzed to date. Id is an HLH protein, but it lacks a basic DNA binding domain. Protein Id is believed to dimerize with other HLH trans acting factors, thereby inhibiting their ability to bind DNA and activate transcription.

The experiments described in this chapter attempt to examine some of the regulatory mechanisms which could control α-actin gene expression during BC3H1 myogenic cell differentiation. As shown earlier, BC3H1 cells sequentially express VSM and SK α-actin during differentiation. We now confirm that expression of the α-actin genes is regulated at least in part at the level of gene transcription, and that the accumulation of α-actin mRNA can be modulated by microenvironmental factors. Transcriptional activity of the α-actin genes is apparently not influenced by methylation status in BC3H1 cells, but α-actin expression parallels the level of myogenin expression, suggesting that myogenin may play a role in activating transcription of α-actin genes in BC3H1 cells.
MATERIALS AND METHODS

CELL CULTURE

Cultivation of BC3H1 myogenic cells was performed as described in Chapter I. To investigate the effect of gene demethylation on BC3H1 cell differentiation, BC3H1 myoblasts were seeded at subconfluent densities, (approximately 100,000 cells per P100 dish), in DMEM 10% FBS. After 24-48 hours, the actively proliferating myoblasts were treated with DMEM 10% FBS containing 5-azacytidine (Sigma) at a concentration of either 3 μM or 10 μM (Taylor and Jones 1979). Forty eight hours later, the cells were re-fed with fresh DMEM 10% FBS and cultivated as per normal. Upon attaining confluence the medium was changed to N2 serum-free medium to induce myoblast cytodifferentiation.
ANALYSIS OF GENE SPECIFIC mRNA EXPRESSION

The preparation of plasmid probes, RNA isolation, and Northern blotting were all performed exactly as described in Chapter I.

DETERMINATION OF GENE TRANSCRIPTIONAL ACTIVITY

Preparation of Biologically Active Cell Nuclei

BC3H1 cells at various developmental stages were harvested according to the following protocol (Ausubel et al. 1991). Dishes were rinsed twice with ice cold, RNase-free PBS, then scraped using a rubber policeman into five ml of ice cold PBS. Scraped cells were centrifuged at 500 x g for 5 minutes at 4° C in a baked Corex tube. The pellet of cells was loosened by vortexing at half-maximal speed for five seconds, then extracted in 4 ml of lysis buffer containing 10 mM Tris-Cl pH 7.4, 10 mM NaCl, 3mM MgCl₂, 0.5% NP-40 (Sigma). Lysis buffer was added while vortexing which continued for 10 seconds. After a 5 minute incubation on ice, an additional 4 ml of lysis buffer was added while vortexing, as above, and the resultant suspension centrifuged at 500 x g for five minutes at 4° C. The supernatant was aspirated off and saved for RNA isolation, while the pellet of nuclei was gently resuspended in 200 μl glycerol storage buffer, (50 mM Tris-Cl pH 7.5, 40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA). Nuclei were transferred to sterile 1.5 ml microfuge tubes, and frozen in liquid nitrogen.
Frozen nuclei were stored at -70° C until the run-on assays were performed. Cytoplasmic RNA was isolated from the supernatant by digestion with Proteinase K (Boehringer Mannheim, final concentration = 0.2 mg/ml) at 37° C for 30 minutes in 1X PK buffer, (1X PK buffer = 0.1 M Tris-Cl pH 7.5, 0.22 M NaCl, 1% SDS, 12.5 mM EDTA). Following phenol extraction, cytoplasmic RNA was isolated by overnight ethanol precipitation at -20° C.

**Preparation of DNA Slot Blots**

Slot blots were prepared using a Minifold II™ slot blot apparatus according to the manufacturer’s instructions (Schleicher & Schuell Inc., Keene, NH). Denatured plasmid cDNA probe was prepared by dissolving the probe in TE, then adding 0.1 volume 3.0 M NaOH and incubating for one hour at 65° C. After cooling to room temperature, an equal volume of 2M ammonium acetate, pH 7.0 was added. Aliquots containing five µg of plasmid DNA were applied to each slot of the slot blot apparatus which was fitted with precut nitrocellulose membranes previously soaked in 1M NH₄OAc. Following application of the plasmid, each well was rinsed once with 0.5 ml of 1M NH₄OAc, and the blot dried under a heat lamp for 10 minutes prior to baking in a vacuum oven for 3 hours at 80° C.
Nuclear Run-On Transcription Assay

200 μl of frozen nuclei were thawed at room temperature and transferred to a 15 ml polypropylene tube. 200 μl of 2X reaction buffer (10 mM Tris-Cl pH 8.0, 5 mM MgCl₂, 0.3 M KCl, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 2.5 mM DTT) was immediately added, followed by 100 μCi of α-[³²P]UTP (approx. 800 Ci/m mole, SP6/T7 grade, Amersham) (Ausubel et al. 1991). The reaction mixture was incubated for 30 minutes at 30° C in a shaking water bath. To terminate the reactions, 0.6 ml of high salt buffer (HSB) containing 0.5 M NaCl, 50 mM MgCl₂, 2 mM CaCl₂, 10 mM Tris-Cl pH 7.4 and 300 units/ml RNase-free DNase I (Boehringer Mannheim) was added. The solution was gently mixed by trituration, and incubated at 30° C for an additional 5 minutes, after which time 200 μl of SDS/Tris buffer, (5% SDS, 0.5 M Tris-Cl pH 7.4, 0.125 M EDTA), and 10μl of 20 mg/ml proteinase K was added. After a 30 minute digestion at 42° C, the mixture was phenol extracted and nucleic acids in the aqueous layer were precipitated by adding 2ml DEPC ddH₂O, 10 μl of carrier E. coli tRNA, and 3 ml of 10% trichloroacetic acid (TCA)/60mM sodium pyrophosphate. After 30 minutes on ice, the precipitated material was filtered onto Whatman GF/A glass fiber filters (Whatman, Maidstone, England), and the filters washed with 5% TCA/30mM sodium pyrophosphate. Filters were transferred to glass scintillation vials and incubated with 1.5 ml DNase I buffer, (20 mM N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) pH 7.5, 5 mM MgCl₂, 1 mM CaCl₂), containing DNase I, (final concentration of 25 μg/ml), for
30 minutes at 37°C. DNase I digestion was quenched by addition of EDTA to 15 mM and SDS to a final concentration of 1.0%. Samples were heated to 65°C to elute RNA from the filters. Released material was removed after 10 minutes and the elution repeated. The collected eluants were digested with proteinase K, (0.3 mg/ml) for 30 minutes at 37°C, then phenol extracted. The aqueous phase was removed to a silanized, RNase free Corex tube, and denatured by the addition of NaOH to a final concentration of 0.2 M. After 10 minutes on ice, the solution was neutralized by adding 1.5 ml 1M HEPES, (free acid, Sigma). RNA was precipitated by adding 0.1 volume 3M sodium acetate and 2.5 volumes ethanol. After overnight precipitation at -20°C, the RNA was pelleted by centrifugation at 10,000 x g for 30 minutes at 4°C. The RNA pellets were resuspended in 1 ml TES solution, (containing 10 mM [N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid pH 7.4, 10 mM EDTA, 0.2% SDS), by shaking at room temperature for 30 minutes. Determination of radioactivity was made by spotting 5 µl aliquots onto Whatman GF/F filters and analysis by scintillation spectroscopy.

Hybridization of Run-On RNA to DNA Probes

An equal amount of radioactivity from each run-on reaction was brought to a 1 ml final volume with TES solution. 1 ml of TES/NaCl solution (TES solution plus 0.6 M NaCl) was added to each aliquot (Ausubel et al. 1991), and the mixture applied to slot blots previously loaded with 5 µg per slot of DNA corresponding to a variety of
gene-specific probes. Hybridization was performed in sealed plastic bags for 36 hours at 65° C. Following hybridization the filters were washed twice for 1 hour with 2X SSC at 65° C. Non-specifically bound single stranded RNA was removed by washing the filters for 30 minutes at 37° C with 2X SSC containing 0.01 mg/ml RNase A, (Boehringer Mannheim). A final 1 hour wash in 2X SSC at 37° C was performed, then the damp filters were wrapped in Saran Wrap and exposed to X-ray film with Cronex Lightning Plus™ intensifying screens at -70° C. Exposures fell within the linear response range of the film.

CHARACTERIZATION OF GENE METHYLATION

DNA Extraction

Genomic DNA was isolated essentially as described (Sambrook et al. 1989). Frozen cell pellets were briefly rinsed in 20 ml of ice cold Tris-buffered saline (TBS, containing 140 mM NaCl, 3 mM KCl, and 25 mM TrisCl pH 7.4), then resuspended in TE pH 8.0 at a ratio of 1 ml TE per 0.5 ml cell volume. 10 ml of DNA extraction buffer containing 10 mM Tris pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS, 0.02 mg/ml RNase A (Sigma) was added per ml of TE and the mixture incubated at 37° C for 1 hour. Proteinase K (Boehringer Mannheim) was mixed gently into the viscous solution to a final concentration of 100 µg/ml. The solution was swirled every 20 minutes for 3 hours in a 50° C water bath and then allowed to cool to room temperature. An equal volume of Tris-buffered phenol, (0.1 M Tris, pH 8.0), was added and
the phases mixed by gentle inversion for 5 minutes. The phases were separated by centrifugation at 2500 x g for 5 minutes at room temperature and the aqueous phase collected using a wide bore micropipette tip and re-extracted with phenol followed by 2 extractions with Tris-buffered:chloroform:isoamyl alcohol (25:24:1) and two final extractions with chloroform:isoamyl alcohol (24:1). DNA was precipitated from the final aqueous phase by adding 0.2 volumes of 10 M ammonium acetate and 2 volumes of 95% ethanol. The DNA precipitated immediately, forming long fibrous strands which were collected using a glass rod. The DNA was rinsed briefly in 95% ethanol, followed by 2 rinses in 70% ethanol, then allowed to dry at room temperature for 15 minutes. The DNA was dissolved in sterile, DEPC-treated ddH2O, and the concentration determined by measuring absorbancy at 260 nm.

**DNA Digestion, Electrophoretic Analysis, and Southern Blotting**

DNA was digested for 20-24 hours using excess amounts of methylation sensitive (Hpa II) and methylation insensitive (Msp I, Eco R1) restriction endonucleases, then ethanol precipitated and stored at -20°C. Endonuclease-digested DNA samples were collected by microcentrifugation, resuspended in DEPC ddH2O, then loaded onto 1X Tris acetate EDTA (TAE, containing 40 mM Tris Acetate and 1 mM EDTA, pH 8.35) gels containing either 0.8% or 1.0% SeaKem GTG™ agarose (Sambrook et al. 1989). Wide bore micropipette tips were used to minimize DNA shearing, and the sample was permitted to sit
for several minutes to allow for even diffusion of the DNA throughout the sample well. Gels were run for 11 to 14 hours at 15 volts constant voltage, (1 volt/cm). Following electrophoresis, gels were stained with ethidium bromide, (EtBr, 5 μg/ml in ddH2O), and the fluorescent DNA visualized and photographed using UV illumination. EtBr was removed by a 30 minute wash in 1mM magnesium sulfate. The gels were washed for 8 minutes in 0.25 M HCl to partially depurinate the DNA, followed by a 30 minute denaturation treatment in 0.5 M NaOH/1.5 M NaCl. Gels were neutralized by soaking in 1.0 M Tris pH 7.5,1.5 M NaCl for 30 minutes, and then soaked in 20X SSC, (3 M NaCl, 0.3 M Sodium Citrate, pH 7.0), for 30 minutes. The DNA was transferred to Duralon™ nylon membranes, (0.45 μM pore size, Stratagene), by overnight capillary diffusion using 20X SSC. DNA was covalently bound to nylon membranes by UV irradiation according to the manufacturers instructions (Stratagene).

The resulting DNA blots (Southern blots) were stored in sealed plastic bags at 4° C until needed for hybridization. To probe DNA for the presence of specific genes, Southern blots were hybridized with gene-specific probes radiolabeled with α-[32P]dATP using a commercially available random oligonucleotide primed labeling system (Multiprime DNA Labeling System, Amersham). The reaction was stopped by the addition of EDTA to final concentration of 20 mM, and unincorporated nucleotides were removed from labeled probe by spermine precipitation as previously described. Blots were prehybridized with a solution of 6X SSC, 50% formamide, 3% SDS, 0.4% DEPC treated non-fat dried milk for at least one hour at 42° C.
Following prehybridization, fresh solution was added containing radiolabeled probe at a concentration of at least 500,000 cpm/ml, (typically 1.0 x 10^7 cpm/ml). Hybridization was performed at 42° C for 36-48 hours, after which time the blot was washed extensively to remove unbound and non-specifically bound probe. Washes were performed as follows: 2X SSC/0.2% SDS 3 x 5 minutes at 22° C, 2X SSC/0.2% SDS 1 x 30 minutes at 63° C, 0.5X SSC/0.2% SDS 1 x 15 minutes at 63° C, 0.1X SSC/0.2% SDS 1 x 15 minutes at 63° C. The final high stringency wash was performed at a temperature two degrees below the melting temperature (Tm) of the probe DNA-genomic DNA duplex, as determined by the following empirical formula (Sambrook et al. 1989):

(Eq. 1)

\[ Tm = 81.5 - 16.61 \log[Na^+] + 0.41(\% \text{GC}) - 820/L - 0.6(\% \text{formamide}) - 1.4(\% \text{mismatch}) \]

where [Na+] corresponds to the concentration of monovalent cations in the hybridization cocktail, %GC corresponds to the percent of G/C base pairs in the duplex, L corresponds to the length of the duplex, % formamide corresponds to the percent formamide in the hybridization cocktail, and % mismatch corresponds to the percentage of mismatched bases between the target and probe DNA sequences.

Washed blots were wrapped in plastic wrap while still slightly damp, and exposed to Kodak XAR-5 film with one or two intensifying screens at -70° C for up to one week. Exposures fell within the linear response range of the film. Southern blots were stripped to permit
reprobing with different cDNA probes. Stripping was performed by pouring boiling 0.1 X SSC/0.2% SDS over the blots and gently agitating the blots for 15 minutes while the solution cooled. This procedure was repeated three times, for a total of four washes, and the blots exposed to film for three days to confirm complete removal of all bound probe.
RESULTS

Strauch and Rubenstein previously presented two-dimensional gel electrophoresis data suggesting that multiple pathways can lead to BC3H1 cytodifferentiation and that expression of certain proteins was influenced by cell culture conditions (Strauch and Rubenstein 1984a). Additional reports demonstrating that VSM α-actin and extracellular matrix protein expression in cultured aortic smooth muscle cells also is affected by culture conditions (Kocher and Gabbiani 1987; Campbell et al. 1989; Liau and Chan 1989), prompted us to further explore the effect of the culture microenvironment on BC3H1 myogenic cell differentiation.

During culture in serum-free, hormone supplemented N2 medium, confluent BC3H1 cells express VSM α-actin and SK α-actin in a sequential manner. Other groups working with the BC3H1 cell line induce differentiation simply by reducing the serum content of the culture medium from 10% FBS to 0.5% FBS (Olson et al. 1983a,b; Olson et al. 1984). In addition, it has been observed that confluent BC3H1 myoblasts will differentiate in serum-containing medium, although the time course is somewhat prolonged (Strauch and
Rubenstein 1984a). Thus, for our study of BC3H1 cytodifferentiation in various culture media, we chose to compare three different media formulations: serum-free, hormone supplemented N2, DMEM supplemented with 0.5% FBS (DMEM 0.5% FBS), and DMEM supplemented with 10% FBS (DMEM 10% FBS). Cells were seeded at subconfluent densities and permitted to grow to confluence in DMEM 10% FBS, at which time the media was changed to one of the three differentiation media listed above. Media was not changed for the remainder of the experiment. Cellular morphology and pattern of α-actin and myogenin mRNA expression were the criteria by which rates of differentiation were compared. As shown in Plate VI, culture media formulation does affect BC3H1 morphologic differentiation.

Subconfluent, exponentially growing BC3H1 myoblasts possess a fibroblastoid appearance (Plate VI, panel A) and at confluence myoblasts assume a more cuboidal or epithelial cell shape (Plate VI, panel B). If at confluence the medium is changed to N2, BC3H1 myoblasts will largely differentiate into spindle-shaped myocytes within two days (Plate VI, panel C). If maintained in DMEM 10% FBS, myoblasts will eventually differentiate but somewhat more slowly than myoblasts treated with N2; compare two day post-confluent myoblasts in DMEM 10% FBS (panel D) with two day post-confluent myoblasts in N2 (panel C). However, serum reduction is not a sufficient stimulus for rapid morphological differentiation, since post-confluent myoblasts cultivated in DMEM 0.5% FBS for two days have not yet begun to elongate (Plate VI, panel E).
PLATE VI. The Cell Culture Media Formulation Affects BC3H1 Morphologic Differentiation

As shown in panel A, subconfluent, rapidly proliferating BC3H1 myoblasts possess a fibroblastoid appearance when grown on tissue culture plastic in DMEM supplemented with 10% fetal bovine serum, (DMEM 10% FBS). At confluence, myoblasts are more cuboidal in shape, (panel B). If at confluence the cell culture medium is changed to serum-free, hormone supplemented N2, a majority of the myoblasts morphologically differentiate into spindle-shaped myocytes within two days, as depicted in panel C. If maintained in DMEM 10% FBS myoblasts will differentiate, but somewhat more slowly than in N2; compare two day post-confluent myoblasts shown in panel D with those in panel C. Serum reduction is not a sufficient stimulus for rapid morphological differentiation, since post-confluent myoblasts cultivated in DMEM supplemented with only 0.5% fetal bovine serum (DMEM 0.5% FBS) have not yet begun to elongate after two days, (panel E). Bar = 17 μM.
Media formulation also affects cell number and the rate of cell division during growth and differentiation. As the data presented in Figure 2 indicates, BC3H1 myoblasts grow rapidly in DMEM 10% FBS until confluence, at which time they become largely contact inhibited and the rate of thymidine incorporation drops dramatically. The rate of thymidine incorporation in post-confluent myoblasts remains comparably low in all three media formulations, but while cell numbers increase slowly for the remainder of the experiment if the cells are maintained in N2 or DMEM 10% FBS, cultivation in DMEM 0.5% FBS results in a significant decrease in cell number. The reduction in cell number, which is apparent in the micrograph in Plate VI panel E, appears to be largely the result of cell detachment from the culture dish, possibly indicating cell death or a change in the adhesive properties of the cells in this medium.

The morphological differences observed between BC3H1 myoblasts undergoing cytodifferentiation in the three different culture media were reflected in the pattern of α-actin mRNA accumulation in these cells. The Northern blot depicted in Plate VII, panel A, was probed with an actin coding region cDNA and reveals that BC3H1 cells differentiating in each of the three media up-regulate the expression of a 1500 nucleotide RNA species that represents predominantly VSM α-actin mRNA (Strauch et al. 1986). Of the three differentiation media, however, N2 induces the most rapid, robust increase in muscle α-actin transcripts, including the SK α-actin mRNA shown in Plate VII panel B. By 4 days post-confluen ce the level of α-actin mRNA in N2 myocytes declines marginally, as is characteristic of late stage BC3H1
Figure 2. The Effect of Media Formulation on BC3H1 Cell
Number and \([^3H]-\)Thymidine Incorporation

BC3H1 myoblasts were seeded at low density in 35 mm dishes and grown in DMEM 10% FBS (dark squares) until confluence. At confluence the media was changed to one of three different media as indicated. Cell counts (A) and tritiated thymidine incorporation (B) were determined at the indicated times. At confluence, BC3H1 myoblasts are largely contact inhibited, although N2 and DMEM 10% FBS permit continued slight additional postconfluent growth. All three media formulations result in comparably low levels of thymidine incorporation. Data is presented as the mean value of duplicate preparations. Standard error values are less than the size of the symbols.
myocytes (Strauch et al. 1986). The level of α-actin mRNA in 4 day DMEM 10% FBS myocytes is roughly equivalent to that observed in 2 day N2 myocytes. Interestingly, when compared to DMEM 10% FBS myocytes, N2 myocytes also contain slightly higher levels of a 2100 nt RNA species corresponding to nonmuscle β- and γ-actin mRNAs. Although α-actin mRNA is induced in DMEM 0.5% FBS myocytes, the level of expression is markedly lower than that observed for the other two media formulations. It is also interesting to note that α-actin mRNA is expressed at low levels in subconfluent myoblasts, then falls to negligible levels in confluent myoblasts before rising again in 2 day post-confluent myocytes (Plate VII, panel A). This pattern is in my experience typical of BC3H1 cell differentiation, and was observed repeatedly in all preparations of confluent BC3H1 myoblasts regardless of clone or passage number. The possible significance of this observation will be discussed below. As seen in Plate VII panel B, the level of SK α-actin mRNA accumulation is greatest in N2 myocytes. Finally, contrary to a published report (Taubman et al. 1989), we are unable to detect CA α-actin mRNA at any stage during BC3H1 cytodifferentiation (Plate VII, panel C). This suggests that the result of Taubman et al may be the result of BC3H1 clonal variation, although differential gene expression caused by different culture media formulation remains a formal possibility since this group used a differentiation promoting medium consisting of DMEM:F12 in a 3:1 ratio supplemented with insulin, transferrin, and selenium. In this regard, it is important to note that another group recently failed to
PLATE VII. The Expression of Vascular Smooth Muscle and Skeletal
Alpha-Actin mRNAs in BC3H1 Cells Is Developmentally
Regulated and Influenced by Media Formulation

Total RNA was isolated from subconfluent or confluent BC3H1
myoblasts in DMEM 10% FBS (lanes S, C), or from, respectively,
confluent BC3H1 myocytes permitted to differentiate in DMEM 10%
FBS (D lanes), serum-free N2 medium (N lanes), or DMEM 0.5% FBS
(M lanes) for varying lengths of time (the number of days is indicated
by the number following the letter). Lanes K and H contain skeletal
and cardiac RNA respectively. Hybridization with an actin coding
region cDNA probe (pHMαA-1) reveals that expression of a 1500
nucleotide mRNA representing predominantly VSM α-actin is up-
regulated in differentiating myocytes, (panel A, arrow). It is
interesting to note that α-actin mRNA is also expressed at low levels
in subconfluent, proliferating myoblasts, then falls to negligible levels
in confluent myoblasts before rising again as myoblasts differentiate
(see text for discussion). Of the three different media used to induce
differentiation, N2 induces a more rapid increase in muscle α-actin
transcripts, including SK α-actin mRNA as depicted in panel B
(probed with pJ3'). CA α-actin mRNA is not detectable at any stage of
BC3H1 differentiation, in any of the three media (panel C, probed with
pHMcA-3'UT-DB)). Approximately 10 μg total RNA was loaded in each
lane. Ethidium bromide fluorescence indicated equivalent amounts of
RNA in each lane (data not shown). Upper and lower arrowheads
adjacent to panel A depict the approximate positions of 28S and 18S
rRNA markers, respectively.
RNA was isolated from subconfluent and confluent myoblasts, and from differentiating myoblasts as described and identified in Plate VII. Myoblasts cultured in all three media express myogenin in a developmentally regulated fashion (arrowhead), suggesting that each formulation is capable of supporting BC3H1 cytodifferentiation. However, myogenin mRNA appears to be slightly more abundant in N2 myocytes. Ethidium bromide fluorescence indicated equivalent amounts of RNA in each lane (data not shown). Image enhancement performed using a Tracor Northern TN8502 Image Analysis System and IPa85 software.
detect CA α-actin in BC3H1 myocytes (Brennan et al. 1990).

BC3H1 cells cultured in all three media transcribe the muscle regulatory factor myogenin (Plate VIII, arrowhead), suggesting that each medium formulation is capable of supporting cytodifferentiation. However, the myogenin expression in N2 myocytes is somewhat greater than that observed in either DMEM 0.5% or DMEM 10% FBS myocytes. Note that the expression of myogenin temporally parallels the appearance of SK α-actin mRNA and the post-confluent up-regulation of VSM α-actin mRNA. From these studies, we can conclude that media formulation does in fact appear to influence the rate of both morphologic and biochemical differentiation of BC3H1 cells, and that optimum cytodifferentiation occurs in hormone supplemented, serum-free N2 medium. The serum-free, defined composition of N2 medium permits myocytes to be manipulated in precise ways without serum interference, and N2 medium was used exclusively as the differentiation promoting medium for the remainder of the experiments described in this dissertation.

The observed changes in α-actin mRNA accumulation during BC3H1 differentiation are consistent with the idea that the regulation of α-actin expression occurs at the level of gene transcription. To determine if in fact α-actin regulation is at the transcriptional level, nuclear run-on assays were performed on nuclei isolated from BC3H1 cells in various stages of differentiation. The results, shown in Plate IX, indicate that VSM α-actin transcription is low in myoblasts, is up-regulated at confluence, and remains elevated in myocytes. SK α-actin transcription is not detectable in subconfluent or confluent myocytes.
Run-on assays were performed on nuclei isolated from BC3H1 cells at various stages of differentiation (indicated as described in Plate VII), and the newly transcribed RNA was hybridized to slot blots containing 5 μg of each of four different clones: exon 1/intron 1 clones of VSM α-actin (p12b, panel A) and SK α-actin (pEMSV688SK, panel B), a full length coding region cDNA clone of myogenin (pMyogenin, panel C), and an "empty" expression vector to serve as a negative control (pEMSV, panel D). The results indicate that VSM α-actin transcription is up-regulated at confluence, while SK α-actin does not increase appreciably until 2 days after exposure to N2 medium. Note that VSM α-actin transcription is significantly greater than SK α-actin transcription in BC3H1 cells at this developmental stage. Myogenin transcription appears to increase 2 days after N2 medium treatment, paralleling the observed increase in SK α-actin transcription and suggesting that myogenin may coordinate SK α-actin gene expression. No nonspecific hybridization is observed to pEMSV alone. Note that coding region cDNA clones generally are less efficient than exon 1/intron 1 clones at detecting newly transcribed RNA.
but is detectable at low levels at the 2 day N2 myocyte stage. Myogenin transcription also does not appear until the 2 day myocyte stage, paralleling the observed increase in SK α-actin transcription and implying not only that myogenin may coordinate SK α-actin gene expression, but that VSM α-actin expression may not require myogenin. Note that the probe used to detect nascent transcripts of myogenin is a coding region cDNA clone and, consequently, is less efficient than exon 1/intron 1 probes at detecting RNA transcribed during the run-on assay. However, the probes for VSM and SK α-actin are both 5’ flanking region probes of comparable length, and for this reason it is not unreasonable to conclude that the run-on results shown are a reasonably accurate reflection of the actual relative rates of transcription of the two α-actin genes. The relative abundance of VSM α-actin mRNA when compared to SK α-actin mRNA in BC3H1 myocytes corroborates the Northern blot analysis discussed earlier (refer to Plate VII).

In contrast to the earlier Northern blot data in which scant VSM α-actin mRNA was detectable in total RNA from confluent BC3H1 myoblasts (Plate VII, panel A), we observe abundant VSM α-actin transcriptional activity in confluent myoblasts using the nuclear run-on assay. Taken together these data suggest that a post-transcriptional control mechanism may regulate VSM α-actin mRNA abundance in early confluent myoblasts. Recall, however, that the peptide mapping studies discussed in Chapter I indicate that VSM α-actin protein expression is up-regulated in confluent myoblasts (Plate I). Clearly, the increased level of VSM α-actin protein observed at confluence
suggests that VSM α-actin mRNA should be present in these cells, and the increased transcription rate in confluent myoblasts suggests that high levels of mRNA should be detectable. One possible explanation for the apparent discrepancy in these data is that at confluence α-actin mRNA is translated immediately after transcription and then rapidly degraded. Reduced transcript half-life could account for the minimal levels of VSM α-actin mRNA detected in confluent myoblast RNA on Northern blots. This explanation requires efficient coupling of transcription and translation so that mRNA pools do not accumulate to detectable levels in confluent myoblasts. As discussed earlier, preliminary data from our laboratory indicates that BC3H1 cells express multiple VSM α-actin mRNAs and that the size distribution of these transcripts changes during myoblast differentiation (B. Min, Ph.D. dissertation). It may be that confluent myoblasts transcribe a special class of VSM α-actin mRNA that is more susceptible to turnover than VSM α-actin transcripts from subconfluent myoblasts and post-confluent myocytes. However, while studies suggesting that CA α-actin transcripts are rapidly translated in vivo (Lyons et al. 1991) lend support to this hypothesis, in vitro translation experiments suggest that VSM α-actin mRNA is not efficiently translated in BC3H1 muscle cells (Strauch and Rubenstein 1984a; Strauch and Rubenstein 1984b). Furthermore, if such a post-transcriptional regulatory mechanism is utilized in confluent myoblasts, there is no evidence to suggest that it is also employed in post-confluent myocytes, since in fully differentiated BC3H1 myocytes the transcriptional activity and mRNA levels of VSM α-actin correlate
nicely. The most straightforward means of determining if this result is in fact indicative of a post-transcriptional regulatory process would be to measure the level of VSM α-actin mRNA in cytoplasmic RNA isolated from the same cells from which nuclei are harvested for run-on experiments. That experiment would tell us if newly transcribed and steady state α-actin mRNA levels are equivalent at a given stage of BC3H1 cytodifferentiation.

A more simple, and perhaps more likely, explanation for the discrepancy inherent in these data is that "confluence", although a critical stage in the establishment of the subsequent BC3H1 differentiation program, is extremely difficult to reproducibly define in practice. Given the difficulty of consistently uniform seeding and growth of cells cultured in vitro, analysis of data obtained from "confluent" BC3H1 cells may be inherently more variable than that obtained from subconfluent and post-confluent cell cultures. Since, as we have seen, confluent BC3H1 cells can differentiate in serum-containing medium, cells harvested at slightly different stages of confluence may in fact vary subtly but significantly in their level of myogenic differentiation. Clearly, further characterization of α-actin gene transcriptional activity immediately before, during, and after the onset of confluence would improve our understanding of the of the regulation of α-actin gene expression at this critical developmental stage.

At the very least then, the nuclear run-on data suggest that α-actin expression in subconfluent myoblasts and in post-confluent myocytes is regulated in part at the level of gene transcription. One of
the means by which gene transcription is itself regulated is by controlling the methylation status of the gene promoter region. Tissue-specific gene expression is associated with undermethylation of CpG sequences upstream from and within the gene. Between 2 and 7% of the cytosine residues in mammalian genomes are methylated, and most of the methyl groups are found in CG doublets. In many genes which are expressed in a tissue-specific manner, the majority of cytosine residues in the gene are constantly methylated. However, a minority of cytosine residues within the gene are methylated in what may be also be described as a tissue-specific manner, i.e. the cytosines are methylated in tissues in which the gene is not expressed, but they are not methylated in tissues in which the gene is transcribed (Cedar and Razin 1990; Lewin 1990). Using the methylation resistant cytosine analog 5-azacytidine (5-azaC), Weintraub and co-workers (Lassar et al. 1986; Davis et al. 1987) were able to activate a myogenic determination program in mouse mesodermal embryonic C3H 10T1/2 fibroblasts. This work, which led to the identification of the myogenic regulatory factor myoD, suggested that gene methylation status may be a regulatory mechanism governing the expression of muscle-specific genes during myogenesis. We therefore attempted to induce "premature" expression of α-actin isoforms in BC3H1 cells by treating them with 5-azaC. Subconfluent BC3H1 myoblasts were grown for 48 hours in DMEM 10% FBS containing either 3 μM or 10 μM 5-azaC, then re-fed with fresh DMEM 10% FBS. At confluence, the medium was changed to serum-free hormone supplemented N2 differentiation medium. Instead of causing precocious induction of myogenesis,
PLATE X.  The Effect of 5-Azacytidine (5-azaC) on BC3H1 Cell
Phenotype: Morphology

In order to investigate the possible role of methylation in the transcriptional regulation of muscle-specific genes, subconfluent BC3H1 myoblasts were grown in DMEM 10% FBS containing either 3 μM or 10 μM 5-azaC for 48 hours, then re-fed with fresh DMEM 10% FBS. At confluence, the medium was changed to serum-free N2. Morphologically, 3 μM 5-azaC treated cells (D-F) appeared to differentiate less rapidly than control cells (A-C). Although subconfluent (A,D) and confluent (B,E) cells exhibited a very similar morphologic appearance, 5-azaC treated cultures (F) contained fewer spindle-shaped myocytes after 4 days in N2 medium than did comparably staged control cultures (C). Bar = 20 μM.
TABLE 1. Ratio of Tritiated Thymidine Incorporation (CPM) to Cell Number (#) in 5-Azacytidine-Treated BC3H1 Myogenic Cells at Various Developmental Stages

<table>
<thead>
<tr>
<th>Stage</th>
<th>CPM / # (± Standard Error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>**Control Cells *</td>
<td></td>
</tr>
<tr>
<td>Subconfluent Myoblasts</td>
<td>0.1980 ± 0.0505 a</td>
</tr>
<tr>
<td>Confluent Myoblasts</td>
<td>0.0161 ± 0.0085 a</td>
</tr>
<tr>
<td>4 Day Post Confluent Myoblasts</td>
<td>0.0083 ± 0.0036 a</td>
</tr>
<tr>
<td><strong>3 μM 5-azacytidine Treated Cells §</strong></td>
<td></td>
</tr>
<tr>
<td>Subconfluent Myoblasts</td>
<td>0.2137 ± 0.0127 a</td>
</tr>
<tr>
<td>Confluent Myoblasts</td>
<td>0.0453 ± 0.0256 b</td>
</tr>
<tr>
<td>4 Day Post Confluent Myoblasts</td>
<td>0.0054 ± 0.0035 b</td>
</tr>
<tr>
<td><strong>10 μM 5-azacytidine Treated Cells</strong></td>
<td></td>
</tr>
<tr>
<td>Subconfluent Myoblasts</td>
<td>0.3151 ± 0.0667 a</td>
</tr>
<tr>
<td>Confluent Myoblasts</td>
<td>0.0256 ± 0.0230 c</td>
</tr>
<tr>
<td>4 Day Post Confluent Myoblasts</td>
<td>0.0119 ± 0.0088 c</td>
</tr>
</tbody>
</table>

a Each value represents the average of four separate experiments
b Each value represents the average of three separate experiments
c Each value represents the average of two separate experiments

* Subconfluent cells were cultivated in DMEM 10% FBS until confluence, at which time the medium was switched to N2 serum-free medium.

§ Treated cells were grown in DMEM 10% FBS + 5-azaC at the concentration indicated for 48 hours, then cultivated in the same manner as control cells.
however, 5-azaC appeared to delay the onset of cytodifferentiation. Morphologically, there were no grossly apparent differences between 5-azaC-treated cells and control cells at subconfluent or confluent densities (compare Plate X, panels D and E with Plate X, panels A and B). However, after four days in N2 differentiation medium, 5-azaC cultures contained fewer spindle-shaped cells when compared to equivalently staged controls (compare Plate X, panel F with Plate X, panel C). In addition, although the ratio of cell number to thymidine incorporation was not significantly different between control cells and drug-treated cells, the mean values for this ratio were generally higher in 5-azaC treated cells than in control cells. Although not conclusive due to the lack of statistical significance, the trend within these data supports the hypothesis that 5-azaC may be inhibiting differentiation and promoting proliferation in treated BC3H1 cells (Table I).

The effect of 5-azaC on α-actin gene expression, as evaluated by Northern blot analysis of total RNA, was not entirely straightforward. In three of the four experiments performed, confluent and postconfluent 5-azaC treated cells contained reduced amounts of all muscle-specific α-actin mRNAs when compared to comparably staged control cells, (Plate XI, lanes 9-13 contain representative RNA samples). In one experiment (Plate XI, lanes 5-8), however, muscle-specific α-actin mRNAs were expressed in confluent 5-azaC myoblasts (see panels B and C, lane 6); no such expression was detected in control cells (Plate XII, lanes 1-4). One consistent observation was that subconfluent 5-azaC cells appeared to express more β- and γ-actin mRNA and more VSM α-actin mRNA than subconfluent control cells.
PLATE XI. The Effect of 5-Azacytidine (5-azaC) on BC3H1 Cell Phenotype: Actin Gene Expression

Northern blot analysis of total RNA from control cells and from cells treated with 3 μM or 10 μM 5-azaC reveals that actin mRNA levels are influenced by exposure to 5-azaC. Interestingly, however, the effect of 5-azaC treatment is not entirely consistent from experiment to experiment. Lanes 1-4 represent total RNA isolated from control cells at the stages indicated using the notation previously described. Lanes 5-8 represent total RNA isolated from 3 μM 5-azaC treated cells at the times indicated. A separate experiment is represented in Lanes 9-13, which contain total RNA isolated from 3 μM (lanes 9,10,12) and 10 μM (lanes 11,13) 5-azaC treated cells at the times indicated. In panel A, hybridization with an actin coding region probe (pHMαA-1) reveals that in one experiment, the level of nonmuscle and muscle actin in confluent 5-azaC myoblasts (lane 6) is increased with respect to comparable control cells (lane 2), while the level of α-actin mRNA in 2 and 4 day 5-azaC myocytes (lanes 7,8) in this experiment is similar to that observed in control cells (lanes 3,4). Panel B represents an identical Northern blot hybridized with p12b, a 5' flanking region VSM α-actin specific probe, while panel C depicts a blot hybridized with pEMSV688SK, a 5' flanking region SK α-actin specific probe. Results from these blots indicate increased expression of both VSM and SK α-actin mRNA in confluent 5-azaC myoblasts when compared to controls, although the level of VSM and SK α-actin mRNA
in 2 and 4 day 5-azaC myocytes is comparable to controls. In three other similar experiments, 5-azaC treatment was observed to reduce the level of α-actin mRNA expression in maturing BC3H1 cells. Representative samples from one of these experiments are in lanes 9-13, and show that both 3 μM and 10 μM 5-azaC drug treatment prevents substantial accumulation of VSM and SK α-actin mRNA in end-stage myocytes compared to controls. Lane 14 contains total RNA from skeletal muscle. Approximately 7.5 μg of total RNA was loaded in each lane. Ethidium bromide fluorescence indicated equivalent amounts of RNA loaded in each lane (data not shown).
These Northern blot results clearly indicate that the level of \( \alpha \)-actin mRNA expression is affected by the action of 5-azaC in a cell density-dependent (or cell stage-specific) manner. A general, stimulatory effect on actin gene expression was noted in subconfluent 5-azaC myoblasts. Furthermore, it appears that 5-azaC promotes maintenance of a dedifferentiated, proliferative state in confluent and post-confluent cells in the majority of experiments performed.

Therefore, in order to determine if \( \alpha \)-actin gene expression in BC3H1 cells is associated with gene demethylation, the methylation status of the SK and VSM \( \alpha \)-actin genes was determined using genomic DNA isolated from both subconfluent and "confluent" myoblasts and from BC3H1 myocytes treated for 5 days with N2 serum-free medium. Genomic DNA was digested for 20-24 hours with an excess of either \textit{Msp} I or \textit{Hpa} II restriction endonucleases. \textit{Msp} I and \textit{Hpa} II are isoschizomers; each recognizes and cuts at the four base palindromic sequence CCGG. However, \textit{Hpa} II will not cleave at sites in which the internal cytosine residue is methylated; \textit{Msp} I is insensitive to the methylation status of the internal cytosine. The Southern blots depicted in Plate XII, panel A and Plate XII, panel B (lanes 1-3) reveal that cleavage of myoblast and 5 day N2 myocyte genomic DNA with \textit{Hpa} II generates identical gene-specific restriction digest patterns upon hybridization with either the VSM 3'UT specific or the SK 5' flanking region specific probes suggesting that the methylation status of the VSM and SK \( \alpha \)-actin genes does not change during BC3H1 differentiation. However, the \textit{Hpa} II digest patterns are significantly different than those obtained by \textit{Msp} I digestion (data for
VSM α-actin shown in Plate XII, panel A, lanes 4-6. SK α-actin data not shown), indicating that some cytosine residues in the VSM and SK α-actin genes are methylated in BC3H1 cells. Thus, it appears that during normal BC3H1 cytodifferentiation, the methylation status of the α-actin genes does not change, and that each gene normally contains several methylated CpG residues. Interestingly, careful comparison of the confluent myoblast Hpa II digestion patterns with those produced by Hpa II digestion of sub-confluent myoblast and post-confluent myocyte genomic DNA indicates that there may be subtle, transient changes in the cytosine methylation pattern of both the VSM and SK α-actin genes in confluent BC3H1 myoblasts. The potential significance of this observation will be addressed in the Discussion.

If cytosine methylation is not used to turn the α-actin genes "on" and "off" in BC3H1 cells, we reasoned that the effect of 5-azaC on α-actin expression observed in Plate XI could be explained if other genes coding for factors influencing α-actin gene expression were transcriptionally activated by the demethylating activity of 5-azaC. Northern blot analysis of total RNA isolated from 5-azaC treated BC3H1 cells reveals that the level of myogenin mRNA in 5-azaC treated cells correlates closely with the observed abundance of α-actin mRNA. In those 5-azaC treated cells which express low levels of α-actin mRNA (refer to Plate XI), the level of myogenin is also low when compared to controls (Plate XIII, panel A, lanes 9-13); in those 5-azaC cells which express α-actin mRNAs precociously, the level of myogenin is also precociously elevated when compared to controls (Plate XIII, panel A, lane 6). These data strongly suggest that myogenin functions as a
PLATE XII. The Methylation Status of the VSM and SK Alpha-Actin Genes Does Not Change during BC3H1 Cytodifferentiation

In order to determine if the methylation status of muscle-specific genes changes during BC3H1 differentiation, genomic DNA was isolated from subconfluent BC3H1 myoblasts (lanes 1,4), confluent myoblasts (lanes 2,5), and 5 day N2 myocytes (lanes 3,6). Overnight restriction endonuclease digestion was performed using the isoschizomers Hpa II (panels A and B, lanes 1-3) and Msp I (panel A, lanes 4-6). Southern blots were probed with a 3'UT VSM α-actin specific cDNA clone (panel A), or with pEMSV688SK, specific for SK α-actin (panel B). In both cases, similar Hpa II restriction endonuclease digestion patterns were obtained in DNA from myoblasts and fully differentiated myocytes (compare lanes 1,2, and 3 within each blot). This suggests that the methylation pattern of the VSM and SK α-actin genes does not overtly change during BC3H1 differentiation. However, comparison of the Msp I digestion patterns with the Hpa II patterns reveals that the VSM α-actin gene is methylated in BC3H1 cells (compare lanes 4-6 with lanes 1-3 in panel A). A similar result was obtained for the SK α-actin gene (data not shown). Ethidium bromide fluorescence indicated equivalent amounts of DNA loaded in each lane (data not shown). Approximate positions of DNA size markers are indicated by the arrowheads to the right of panels A and B.
PLATE XIII. The Expression of Muscle Regulatory Factors in BC3H1 Cells Is Affected by Exposure to the Demethylating Agent 5-Azacytidine (5-azaC)

Panels A and B both represent Northern blots identical to those depicted in Plate XI (information about RNA samples is described in the legend to Plate XI). Note that myogenin expression in control BC3H1 cells is first detected in post-confluent 2 day N2 myocytes. In one 5-azaC experiment, myogenin expression was induced in confluent myoblasts (panel A, lane 6); these same 3 μM 5-azaC confluent myoblasts also expressed SK α-actin (Plate XI, panel C, lane 6) and VSM α-actin (Plate XI, panel B, lane 6). In other 5-azaC experiments however, myogenin expression was reduced in 5-azaC myocytes with respect to controls (panel A, lanes 12,13). These same cells also contained lower levels of VSM and SK α-actin mRNAs compared to controls (Plate XI, panels B and C, lanes 12,13). In all experiments, 5-azaC exposure resulted in a uniform increase in the level of protein Id mRNA (panel B). The level of protein Id expression in 5-azaC myoblasts (lanes 5, 9) was greater than in control myoblasts (lane 1). Note that Id expression is down-regulated in confluent myoblasts and in myocytes, although the level of Id mRNA remains marginally greater in confluent 5-azaC myoblasts than in confluent control myoblasts. Ethidium bromide fluorescence indicated equivalent amounts of RNA loaded in each lane (data not shown).
PLATE XIII
To determine if the methylation status of the Id gene or myogenin gene changes during BC3H1 cell differentiation, genomic DNA was isolated from BC3H1 cells at various stages of differentiation and assayed as described in Plate XIII. Southern blots were probed with either a full length protein Id cDNA (panel A) or a full length myogenin cDNA (panel B). The results indicate that the DNA methylation status of the Id gene may in fact change during BC3H1 cytodifferentiation. A 1.8 kB fragment that is produced by Hpa II digestion of myoblast DNA (indicated by the arrow to the left of panel A, lane 1) is not present in a Hpa II digest of myocyte DNA (compare lanes 1 and 3). Msp I digestion of myoblast DNA (lane 4) gives a banding pattern similar to that observed in a Hpa II digestion of myocyte DNA, suggesting that the absence of the band in lane 3 is not due to incomplete digestion but rather a change in the DNA methylation status of the Id gene during cell differentiation. In contrast, the methylation status of the myogenin gene apparently does not change (panel B). Hpa II digestion patterns are equivalent in myoblasts and myocytes, (the arrow to the left of panel B indicates the position of the single faint band appearing in lanes 1, 2, and 3). Ethidium bromide fluorescence indicated equivalent amounts of DNA loaded in each lane (data not shown). Approximate positions of DNA molecular weight markers are indicated by the arrowheads to the right of panels A and B.
factor which induces α-actin gene expression during BC3H1
cytodifferentiation. Identical Northern blots were also probed with a
cDNA for protein Id, an HLH protein which inhibits the differentiation
of skeletal muscle cells. In both experiments depicted in Plate XIII,
subconfluent 5-azaC treated myoblasts express increased levels of
protein Id mRNA when compared to controls (panel B, lanes 5 and 9).
The level of protein Id mRNA remains slightly elevated above control
levels in confluent 5-azaC myoblasts, but Id mRNA is not detectable in
postconfluent 5-azaC myoblasts. On the basis of the Northern blot
analyses, it is tempting to speculate that increased expression of
protein Id results in delayed onset of myogenic differentiation in 5-
azaC treated BC3H1 cells, and that α-actin expression is precociously
up-regulated only in those 5-azaC treated cells which also express
increased levels of myogenin. These data therefore suggest that a
balance between Id and myogenin levels may dictate the level of α-
actin mRNA expression in BC3H1 cells. To determine if either
myogenin or Id expression is associated with gene demethylation,
genomic DNA was analyzed for the presence of altered cytosine
methylation at CCpGG sites. Analysis of the methylation status of the
myogenin gene during BC3H1 differentiation reveals that myogenin
methylation status apparently does not change during differentiation
(Plate XIV, panel B). The Msp I digestion pattern (not shown) was
equivalent to the Hpa II digestion pattern, indicating that the
myogenin gene is normally undermethylated in BC3H1 cells. Analysis
of the Id gene, however, reveals that Id gene methylation status of may
in fact correlate with transcriptional activity (Plate XIV, panel A.
A 1.8 kilobase *Hpa* II DNA digestion fragment is present in genomic DNA from subconfluent myoblasts. This fragment is not detectable in a *Hpa* II digest of genomic DNA from 5 day N2 BC3H1 myocytes, suggesting that the cytosine bases surrounding this fragment have undergone a change in methylation status during BC3H1 cytodifferentiation.

These data suggest that persistent up-regulation of Id in 5-azaC treated BC3H1 cells may be explained by the demethylating effects of 5-azaC. Since the myogenin gene is apparently normally undermethylated in BC3H1 cells, the positive effect of 5-azaC on myogenin mRNA levels in BC3H1 cells might be due to altered expression of novel myogenic regulatory factors which control cell determination and/or differentiation and which are capable of activating myogenin transcription. The somewhat variable results obtained from the 5-azaC experiments may be due to the random nature of 5-azaC incorporation in the BC3H1 culture population, or variable susceptibility to the drug effect within the cell population. Since DNA replication is required for 5-azaC uptake, clonal variation in the rate of replication/cell cycle progression of the BC3H1 cells used in these experiments might help explain the differences observed between experiments.

When taken together, the results presented in this chapter suggest that α-actin gene expression during BC3H1 cytodifferentiation may be dependent less on genetically encoded developmental signals than on epigenetic signals. These epigenetic signals can originate from the cellular microenvironment, and appear to be transmitted to
the gene via the action of inducible \textit{trans}-acting factors. Identifying and understanding the nature of these signals will require isolation of the \(\alpha\)-actin gene promoters. Our laboratory has successfully cloned and sequenced the VSM \(\alpha\)-actin upstream regulatory region, and the results of preliminary experiments characterizing the responsiveness of the promoter to selected epigenetic inputs is discussed in the third chapter of this dissertation.
DISCUSSION

The results presented in this chapter suggest that α-actin expression in developing BC3H1 myogenic cells is regulated at the level of gene transcription. In subconfluent myoblasts, which express low levels of VSM α-actin protein and mRNA, the rate of gene transcription is similarly low. SK α-actin protein, mRNA, and nascent transcripts are undetectable in myoblasts. In post-confluent myocytes, increased transcription of the VSM α-actin gene corresponds to the high levels of VSM α-actin protein and mRNA detected. In addition, the (relatively) lower level of SK α-actin gene transcription detected in post-confluent BC3H1 myocytes corresponds to the comparatively low level expression of SK α-actin protein and mRNA in these cells. The data also are suggestive of possible post-transcriptional regulation of VSM α-actin expression in confluent myoblasts, since accumulation of mRNA encoding this actin isoform is somehow inhibited despite a relatively high rate of transcription. These observations may have physiological significance in regard to the cellular changes which accompany myogenesis. For example, just prior to myotube formation, skeletal myoblasts apparently reorganize their cytoskeleton resulting
in loss of microfilamentous components (Fulton et al. 1981). This may happen in anticipation of constructing a new sarcomeric cytoskeleton with muscle-specific protein isoforms. Despite being incapable of forming myotubes, BC3H1 cells may still respond (albeit incompletely) to the signals which indicate the conditions are right for cell-cell fusion. Perhaps newly confluent BC3H1 myoblasts transiently down-regulate expression of the VSM α-actin isoform in anticipation of fusion and the inauguration of a new pattern of α-actin gene transcription.

These data also strongly suggest that although the overall pattern of isoform switching during BC3H1 differentiation remains the same regardless of media formulation, the microenvironment may subtly influence α-actin expression during BC3H1 differentiation. Confluent BC3H1 myoblasts can differentiate in either of the three media studied, including medium containing 10% FBS, but serum-free, hormone-supplement N2 defined medium appeared to optimally induce differentiation. Serum reduction is not a sufficient condition for rapid differentiation, however, since myoblasts differentiated poorly in DMEM containing only 0.5% FBS. This indicates that a factor or factors common to FBS and the additives in N2 medium promote or are at least permissive for the efficient differentiation of BC3H1 cells in vitro. Serum is known to contain a variety of growth and attachment factors (Schubert 1984), and although a number of growth factors have been shown to inhibit BC3H1 cytodifferentiation (Glaser and Wice 1989; Spizz et al. 1986; Wice et al. 1987), BC3H1 cells are capable of morphological and biochemical differentiation even
in the presence of 10% FBS (Strauch and Rubenstein 1984a, this study). It is important to note, however, that two-dimensional protein gel electrophoresis analysis of extracts of BC3H1 myocytes permitted to differentiate in 10% FBS and in N2 reveals that the pattern of protein expression differs under the two distinct conditions (Strauch and Rubenstein 1984a), implying that the pathways leading to cytodifferentiation, or the endpoints attained, may differ according to media formulation in ways that we have yet to define. Cellular attachment factors present in serum may form complexes with secreted macromolecules from BC3H1 cells (substrate associated material, or SAM, and resuspended particulate material, or RPM, cf. Strauch et al. 1991) which ultimately results in the establishment of differentiation-permissive substrate conditions. Moreover, mitogens present in serum may be inactivated by heparin sulfate glycosaminoglycans secreted by developing cells (Fritze et al. 1985).

Although the evidence presented here supports the idea that α-actin expression is regulated at the level of gene transcription, the mechanism of action is apparently not overtly controlled by α-actin gene methylation. The assay used to determine gene methylation status employed methylation sensitive restriction enzyme digestions of BC3H1 genomic DNA in combination with Southern blotting analysis using gene-specific probes. The data indicate that there is no major change in the methylation status of either the VSM or the SK α-actin genes during BC3H1 differentiation. There is, however, some evidence to suggest that the methylation pattern of the SK and VSM α-actin genes is transiently altered during the transition state from
subconfluent myoblasts to differentiating myocytes, i.e in "confluent myoblasts". In order to definitively characterize the actin gene methylation patterns during BC3H1 cytodifferentiation, however, these experiments should be repeated in order to detect fragments smaller than 1000 bp which might be generated by the restriction digest. In addition, the probe used to detect VSM α-actin gene sequences was directed against the 3'UT region of the gene. Although the VSM α-actin gene 3'UT may in fact have some transcriptional regulatory role as discussed earlier, it would have been preferable to have used a 5' flanking region-specific probe. CpG islands and other CpG doublets putatively involved in methylation dependent gene regulation are typically located at the 5' end of a gene in the promoter region. It is therefore unlikely that a 3'UT probe would detect all Hpa II digested genomic DNA fragments containing the 5' flanking region. However, Southern blots identical to those shown in Plate XII and probed with a VSM α-actin coding region cDNA provided no evidence of differential gene methylation for any actin gene (data not shown). Taken together, these results suggest (but do not prove) that the methylation status of the VSM and SK α-actin genes in proliferating myoblasts is identical to that in fully differentiated myocytes.

The Southern blot data also suggest, however, that in confluent myoblasts the α-actin genes may undergo a transient change in their methylation status. Inspection of the methylation assay Southern blots depicted in Plates XII and XIV reveals that the Hpa II digestion pattern of the VSM and SK α-actin genes in confluent myoblasts is subtly different with regard to band intensity when compared to the
α-actin gene-specific digestion patterns observed for subconfluent myoblasts and 5 day myocytes. One hypothetical mechanism by which such a transient change in α-actin gene methylation status could occur is that the VSM and SK α-actin genes become undermethylated at the onset of BC3H1 cytodifferentiation during transcriptional activation. During transcriptional activation, the promoter regions become occupied with RNA polymerase/transcription complexes. Once activated, the genes can continue to transcribed, despite subsequent remethylation, if the promoter regions remain perpetually occupied (and therefore resistant to condensation into an inactive heterochromatic configuration). If this proposed sequence of events actually occurs in confluent BC3H1 myoblasts, then the α-actin genes should become sensitive to digestion with DNase I at confluence. It has been shown that the SK α-actin gene does become sensitive to DNase I digestion upon myogenic differentiation in the L8 cell line (Carmon et al. 1982). Determination of the DNase I sensitivity of the VSM and SK α-actin genes throughout BC3H1 cytodifferentiation will therefore help to establish the potential significance of any transient change in α-actin gene methylation status with regard to transcriptional activation.

In addition to determining the methylation status of the α-actin genes, we employed a cell biological approach to investigate the role of gene methylation in BC3H1 differentiation. Subconfluent BC3H1 myoblasts were exposed to the methylation resistant cytidine analog 5-azacytidine. Since 5-azaC treatment of C3H 10T1/2 embryonic fibroblasts initiates myogenesis in a significant fraction of the cells
(Taylor and Jones 1979), we reasoned that by stably demethylating BC3H1 cells we might induce precocious cytodifferentiation. Instead, BC3H1 myoblasts cultured in either 3 μM or 10 μM 5-azaC were in general noticeably delayed in their ability to differentiate upon reaching confluence. In three of the four 5-azaC experiments performed, VSM and SK α-actin mRNA levels were lower than in comparable control cells. In one experiment, however, both VSM and SK α-actin mRNAs were expressed in abundance in confluent myoblasts. Interestingly, in the 5-azaC treated cells expressing reduced levels of α-actin mRNA, the level of protein Id was increased in early stage cells, while the level of myogenin was reduced in late stage cells. Protein Id is a negative regulator of HLH DNA binding proteins. Itself an HLH protein, but lacking a basic DNA binding region, Id acts by dimerizing with HLH proteins and preventing their interaction with DNA. These observations are consistent with the hypothesis that increased levels of protein Id in 5-azaC treated cells might delay the onset of BC3H1 cytodifferentiation by inhibiting the expression of muscle-specific genes, (possibly including myogenin and the α-actin genes), which are transcriptionally regulated by HLH trans-acting factors.

In one 5-azaC experiment precocious expression of both VSM and SK α-actin was detected in confluent myoblasts. Interestingly, increased Id expression was also detected in myoblasts from that experiment. Compared to confluent control cells, however, myogenin mRNA and both VSM and SK α-actin mRNAs were abundantly expressed in confluent 5-azaC myoblasts. The correlation of
precocious expression of α-actins with premature high-level expression of myogenin suggests that myogenin is involved in α-actin transcriptional activation. Moreover, the stimulatory effect of myogenin may override any inhibitory effect of protein Id in these 5-azaC myoblasts. These experiments are far from definitive, and yield no information regarding the direct or indirect nature of myogenin action; myogenin may directly trans-activate α-actin expression, or it may activate another muscle regulatory factor which in turn activates α-actin expression. Similarly, it is impossible to know when the differentiation inhibitory effect of Id is dominant or if it is affected by myogenin expression. Nevertheless, these data appear to indicate that myogenin and Id play dynamic roles in coordinating α-actin expression during BC3H1 myogenic cell differentiation. Transient transfection experiments with VSM α-actin promoter region/reporter gene constructs and myogenin/Id expression vectors will be performed in order to ascertain if either exogenous myogenin or Id functions in VSM α-actin transcriptional regulation.

One reason the 5-azaC experiments are somewhat difficult to interpret is that the effects of 5-azaC are random, depending on which loci incorporate the analog during DNA replication (although there is evidence to suggest that 5-azaC acts by actually binding to and sequestering a maintenance methylase, thereby increasing its effective concentration and promoting undermethylation of cytidine residues throughout the genome, Michalowsky and Jones 1989a). In addition, 5-azaC has pleiotropic effects not only on gene transcription but on several aspects of cellular metabolism. The ratio of thymidine
incorporation to cell number was generally greater in 5-azaC treated cells than in control cells, (although the difference was not statistically significant), and suggests that at least there appear to be no deleterious or lethal effects of 5-azaC on BC3H1 cells in culture. The design of the experiment also contributed to the difficulty in interpreting the data. Rather than reseeding 5-azaC treated cells at clonal densities and observing the phenotype of several different 5-azaC clonal populations, treated cells were permitted to grow to confluence and harvested as a heterogenous population. Thus, within the population there may be many different genes affected by 5-azaC, but only the genes whose transcription is altered frequently or dramatically within the population will be detected in the subsequent Northern blot analyses. Within the culture population, some cells may be susceptible to 5-azaC effects while others may not. This variability may not be inconsequential; skeletal myoblasts within the same culture have been shown to be phenotypically variable with respect to their myoD phenotype (Tapscott et al. 1988), and there is evidence for diversity within BC3H1 clonal populations since not all cells undergo differentiation in N2 (refer to Plate XVI, Chapter III), or when placed on SAM in N2 (Strauch et al. 1991).

The observation that mRNA levels of Id (consistently) and myogenin (in one experiment) were elevated in 5-azaC treated BC3H1 cells indicates that perhaps the transcription of these myogenic regulatory genes is controlled by DNA methylation. The DNA methylation assay confirmed that Id expression may in fact be regulated by gene methylation. Additional support for this hypothesis
could be obtained by determining the DNase I sensitivity of the Id gene in myoblasts and myocytes; if methylation inhibits Id transcription in myocytes then one would expect the gene to be DNase I insensitive in these cells (Keshet et al. 1986). Although these experiments provide no strong evidence for direct regulation of the α-actin or myogenin genes by methylation, based on the results of the methylation assay we can infer that the myogenin gene is undermethylated in BC3H1 cells, suggesting that it may be under the control of other regulatory factors whose regulation is perhaps methylation dependent.

There is no real evidence that housekeeping genes in somatic cells are dynamically turned on and off by altering their methylation state (Cedar 1988; Cedar and Razin 1990). Rather, it is likely that cytosine methylation acts merely to maintain transcriptionally silent genes in that state, and that methylated genes can be activated and transcribed in spite of heavy CpG methylation (Cedar 1988; Cedar and Razin 1990). There is, however, considerable evidence that genes expressed in a developmentally regulated manner are governed by methylation. Yisraeli has shown that the transcription of a transfected α-actin gene in fibroblasts is dependent on methylation status (Yisraeli et al. 1986, Paroush 1990). There is some evidence that expression of the myoD gene is associated with demethylation in 5-azaC 10T1/2 cells (H. Weintraub unpublished data, and Michalowsky and Jones 1989b), and the expression of protein Id, which may act to regulate the timing of the onset of muscle differentiation, apparently also is affected by methylation (this study). Although apparently normally
demethylated in BC3H1 myoblasts, the myogenin gene is not transcriptionally active until after BC3H1 myoblasts have achieved confluence (refer to Plate IX). Since myogenin is a tissue-specific regulatory factor, it is not unreasonable that myogenin should be demethylated in BC3H1 myoblasts. As a muscle determination gene, myogenin likely would be demethylated in all muscle cell lineages. This result implies, however, that undermethylation does not so much turn the myogenin gene "on" as it makes the gene "transcriptionally competent". The myogenin Southern blot data, in conjunction with the 5-azaC experimental data, therefore suggest that myogenin expression may be dependent upon other regulatory factors whose regulation may also be methylation dependent. Such a gene might be activated by 5-azaC in BC3H1 cells and in turn activate myogenin transcription. One candidate gene for this role is myf 5, which has been found to precede myogenin and myoD expression in embryonic somites (Bober et al. 1991). Another possibility is myoD, which has been shown to activate myogenin transcription, (although the myoD gene may be refractory to 5-azaC activation in BC3H1 cells, Brennan et al. 1990). The candidate gene clearly cannot be identified on the basis of speculation, but it is also evident that the cascade of genetic and epigenetic interactions leading to BC3H1 myogenic cell differentiation and α-actin gene transcription is not governed by a single myogenic determination factor.
CHAPTER III

INTRODUCTION

Results presented in the first two chapters of this dissertation suggest that the expression of α-actin genes during BC3H1 cell differentiation appears to be regulated at the level of gene transcription. This chapter describes experiments which aim to elucidate the means by which actin gene transcriptional activity is regulated in BC3H1 cells. In the absence of strong evidence for regulation of actin isoform switching by permanent changes in gene methylation status, we became interested in the role of trans-activation factors in actin gene expression.

Promoters may be defined as DNA sequences which regulate initiation of gene transcription in cis. Promoters which govern transcription of genes that encode protein (i.e. those genes which are first transcribed to mRNA by RNA polymerase II) are typically located upstream from the transcription start site and contain multiple cis-acting regulatory sequences which are recognized by trans-activation
factors. These factors recognize and bind to specific DNA sequence elements within the promoter, and initiate the formation of a transcription complex which ultimately binds RNA polymerase II prior to the initiation of transcription (Lewin 1990).

The first evidence that transcriptional activating factors are involved in myogenic differentiation was obtained by studying muscle cell: nonmuscle cell fusions called heterokaryons (Blau et al. 1985). It was observed that upon cell fusion the nonmuscle cell nucleus began transcribing mRNA for muscle-specific genes, suggesting that factors responsible for activating muscle-specific gene transcription in the muscle cell were diffusing into the nonmuscle cell nucleus and initiating transcription of previously inactive genes. Additional evidence that muscle differentiation is regulated by transcription factors was obtained by the observation that myoD can force expression of muscle-specific genes in nonmuscle cells (Weintraub et al. 1989). Similar experiments have permitted the identification of at least four other muscle regulatory factors, including myogenin (Wright et al. 1989), myf 5 (Rhodes and Konieczny 1989), mrf4/herculin/ myf 6 (Braun et al. 1989), and myd (Pinney et al. 1988).

Both myogenin and myoD have been shown to be nuclear phosphoproteins. Transfection of cells with expression vectors indicates that both of these DNA binding proteins can activate the muscle-specific muscle creatine kinase (MCK) enhancer by binding to a conserved sequence known as an E box (consensus sequence CANNTG, Murre et al. 1989). Myogenin and myoD are both members of the helix-loop-helix (HLH) family of muscle regulatory factors, and
both have been found to function by forming either homodimers or heterodimers with the ubiquitous HLH transcription factor E12. Heterodimers have been determined to have greater DNA binding and transcriptional activating activity than homodimers, and it is believed that the heterodimer is the principal physiologically active species within the cell (Lassar et al. 1991; Brennan and Olson 1990; Davis et al. 1990; Murre et al. 1989 a,b) This mode of transcriptional activation by dimerization allows other HLH proteins to regulate myogenin and myoD transcriptional activity by competing for available E12 (Jones 1990).

Protein Id is capable of inhibiting the DNA binding activity of myoD and has been shown to inhibit myogenic differentiation (Benezra et al. 1990). The interaction of myogenin and Id has not yet been investigated. The discovery and characterization of Id suggests that myogenesis and the action of HLH myogenic factors is under negative control in nonmuscle cells. In other words, HLH myogenic factors which act as positive transcriptional activators may be prevented from functioning in undifferentiated cells by the presence of an inhibitory factor (negative regulation). In fact, recent somatic cell hybrid experiments show that myoD is normally repressed in differentiated nonmuscle cell types by a putative trans-acting factor (Thayer and Weintraub 1990). Further evidence for negative regulation of myogenic differentiation in nonmuscle cells was obtained by Schafer et al (1990), who determined that when transfected into liver cells (which are nonmesodermal and nonmuscle) both myogenin and myoD are unable to initiate muscle-specific gene transcription. Exogenous
myogenin and myoD can activate transcription in fibroblasts, as we have seen. When fibroblasts are fused with liver cells stably transfected with myoD, both the fibroblast and the liver cell are able to transcribe muscle specific proteins. These data therefore also indicate that fibroblasts contain factors which are required in addition to myoD itself in order to activate the myogenic program in the liver nuclei. In addition, Peterson et al. (1990) have characterized a non-differentiating muscle cell line called the NFB mutant, which actually represses expression of HLH myogenic regulators by way of an as yet uncharacterized diffusible factor.

As these studies suggest, negative regulation may apply not only to the myogenic regulatory factors themselves but to the muscle-specific genes which they activate. In this regard, it is interesting to note that not only is the serum-inducibility of nonmuscle β-actin gene expression dependent on an intact "serum response element" (found in the promoter region of the gene, and discussed later), but also that upon inhibition of protein synthesis with cycloheximide, β-actin mRNA expression becomes "super-inducible" (Subramaniam et al. 1989; Elder et al. 1988; Rivera et al. 1990). These data imply that the β-actin gene, perhaps like its muscle-specific α-actin counterparts, is constantly under some form of negative regulation. In fact, negative control elements have been identified within the upstream promoter region of the SK α-actin gene (Chow and Schwartz 1990).

The promoters of both the CA and SK muscle α-actin genes have been well characterized, and much has been learned about the factors
which bind to specific sequences within the promoter and regulate the transcriptional activity of these genes. It is interesting to note that the CA and SK \( \alpha \)-actin promoters share similar, highly conserved sequence motifs referred to as CArG elements, (for CC(A-T rich)GG sequences). CArG elements, also called CArG boxes, have a consensus sequence of CC(A/T)\(_6\)GG. Curiously, however, the CArG element is nearly identical to the serum response element (SRE) found in a variety of serum-responsive genes such as the c-fos gene. In addition, CArG binding factor is identical to serum response factor (SRF; Boxer et al. 1989). This raises the question of why sequences which are involved in regulating a variety of serum-response genes should also be involved in transcription of muscle specific \( \alpha \)-actin genes. The answer, apparently, is that muscle-specific activation of \( \alpha \)-actin gene transcription depends on the interaction between a number of diverse transcription factors. For example, transcription of the CA \( \alpha \)-actin gene has been shown to require the interaction between myoD, the ubiquitous transcription factor Sp1, and CArG binding factor (Sartorelli et al. 1990). A number of different factors with affinity for the conserved regions of the SK and CA \( \alpha \)-actin promoters have been identified and are being characterized (Walsh 1989; Walsh and Schimmel 1987; Walsh and Schimmel 1988). Intuitively it is easy to understand how multifactorial transcription regulators can result in dynamic regulation of the transcriptional status of several genes whose products are also likely to be integrated and connected at some level within the cell.
Our understanding of the promoter region of the VSM α-actin gene is far less advanced than that of the SK and CA α-actin gene promoters. To that end, members of our laboratory have cloned, sequenced, and partially characterized functional properties of the 5’ flanking region of the mouse VSM α-actin gene. From the start of transcription to a point 206 base pairs upstream (-206, or +861 as numbered in Figure 4), the mouse sequence is 73% and 89% homologous to the chicken and human 5’ flanking/UT regions, respectively (Min et al. 1990; Min et al. 1988; Carroll et al. 1986; Reddy et al. 1990). In particular, two putative cis-acting regulatory CArG elements are 100% conserved between mouse, human, and chicken with respect to sequence and distance from the start of transcription (CArG boxes A and B as diagrammed in Figure 3). A third upstream CArG box (box C) was completely conserved between mouse and human, while a fourth CArG box (box D) was unique to mouse. Interestingly, the orientation of the ‘C’ CArG box was inverted with respect to boxes A, B and D. Transient transfection studies have shown that the first three CArG boxes are required for "core promoter" activity in BC3H1 cells, while the fourth CArG box may act in cis as a repressor element preventing expression in nonmuscle cells. Also of note are six "E boxes" which are located farther upstream from the CArG boxes. These E boxes may serve as DNA binding sites for trans-acting regulatory factors containing the HLH structural motif.

The goal of the present study, therefore, was to further characterize the murine VSM α-actin promoter region and to
determine if particular promoter domains might mediate the transcriptional response to various epigenetic signals generated as BC3H1 myoblasts progress through the cytodifferentiation process.
CELL CULTURE

Cultivation of BC3H1 myogenic cells was performed as described in Chapter I. Substrate associated material, (SAM) was prepared from BC3H1 myocytes cultivated in N2 medium for 6 days (Strauch et al. 1991). Myocytes were non-enzymatically detached from the culture dishes as a continuous sheet of cells by incubating monolayers with 0.1% EDTA in Dulbecco's calcium and magnesium free phosphate buffered saline (PBS) for 5-minutes at room temperature (22°C). SAM-coated dishes were rinsed 3 times with PBS, then seeded with BC3H1 myoblasts suspended in N2 differentiation medium at a density of approximately 1 x 10^5 cells/100 mm dish.
DETERMINATION OF MOUSE VSM α-ACTIN PROMOTER ACTIVITY

Transient Transfection of BC3H1 Cells

BC3H1 cells were transiently transfected with VSM α-actin promoter region/bacterial chloramphenicol acetyl transferase (CAT) fusion vectors (shown in Figure 4) using calcium phosphate precipitation according to the following protocol (Ausubel et al. 1991). Cells selected for transfection were re-fed with fresh medium three to four hours prior to addition of the plasmid DNA. Each 100 mm dish of cells received 5 μg of plasmid DNA in a solution containing 0.125 M CaCl₂, and 1X HEPES buffered saline (HEBS, containing 280 mM NaCl, 50 mM HEPES, 1 mM Na₂HPO₄·7H₂O, pH 7.12). After 16 hours at 37° C, DNA/CaCl₂ complexes were removed with one or two washes with warm PBS, and the cells re-fed with fresh medium. Cells were harvested for CAT assay after a 48 to 72 hour recovery period. Cotransfections were performed essentially as described above, except that 5 μg of each plasmid was added per 100 mm dish of cells.

To determine the effect of cell density on promoter activity, cells were transfected at subconfluence, (30-40 % confluent), confluence, or 4 days post-confluence. In other experiments, subconfluent BC3H1 cells were transfected and allowed to recover for 24 hours, then non-enzymatically harvested using Nonenzymatic Cell Dissociation Solution (Sigma) and reseeded at half their original density. To half of the dishes, additional non-transfected myoblasts were added in order to increase cell density and permit the formation
of significant cell-cell contacts. Both "cell contact negative" and "cell contact positive" cells were harvested 48 hours after replating. Finally, to examine the effect of substrate composition on promoter activity, subconfluent transfected cells were replated at subconfluent densities onto either untreated tissue culture plastic or onto substrate attached material (SAM) that was prepared from 6 day BC3H1 myocytes as described above. Transfected cells were maintained in N2 serum-free medium for seven days prior to harvesting. In some cases, fetal bovine serum (FBS) was added to a final concentration of 10% on day 7 to observe the effect of serum stimulation on promoter activity. For control dishes, 1ml of fresh N2 serum-free medium was added. Cells were harvested 16-18 hours later.

**Promoter : Reporter Gene Assay**

Transfected cells were washed twice with five ml of ice cold PBS, then scraped into one ml of TEN solution (40 mM Tris-Cl pH 7.5, 1 mM EDTA pH 8.0, 150 mM NaCl), and pelleted by microcentrifugation for one minute. The cell pellet was resuspended in 0.25 M Tris pH 7.5, and the cells lysed by four cycles of freezing in ethanol/dry ice and thawing at 37° C (Ausubel et al. 1991). Cellular debris was removed by microcentrifugation for five minutes at room temperature. Endogenous deacetylases in the supernatant were inactivated by heating at 65° C for 10 minutes, then cleared from the supernatant by microcentrifugation for two minutes at 22° C. Aliquots were removed from the supernatant for determination of protein
concentration using the bicinchoninic acid (BCA) colorimetric microprotocol, according to the manufacturer's instructions, (Pierce, Rockford, IL). The supernatant was quick frozen in ethanol/dry ice and stored at -20° C until required. To assay for CAT activity in different cellular extracts, equivalent amounts of total protein were incubated for four hours at 37° C in a reaction cocktail containing 0.25 M Tris pH 7.5, 5.3 mM acetyl coA, (sodium salt, Sigma), and 0.1 μCi D-threo-(dichloroacetyl-1-14 C)-chloramphenicol, (57 mCi/mmol, Amersham) (Ausubel et al. 1991). Typically, a volume of extract containing 300 - 1000 ng of total protein was assayed in a reaction volume of 150 μl. Radiolabeled chloramphenicol and its acetylated derivatives were extracted from the reaction mixture with 1 ml ethyl acetate. Following a five minute microcentrifugation period at room temperature, the supernatant was removed and dried in a Speedvac™ (Savant Instruments, Hicksville, NY) for one hour. The residue was resuspended in 30 μl ethyl acetate, and spotted onto a Whatman silica gel thin layer chromatography (TLC) plate. The chloramphenicol and its acetylated derivatives were separated by TLC using chloroform : methanol (19 : 1) as a solvent. After 45 minutes the plate was removed from the solvent, allowed to dry briefly at room temperature, then wrapped in plastic wrap and exposed to Kodak XAR-5 film for periods ranging from 24 to 72 hours. Exposures fell within the linear response range of the film.
RESULTS

The data presented in Chapter II of this dissertation indicate that cytosine methylation does not appear to serve as a simple, self-contained regulatory "switch" governing transcription of the muscle actin genes during BC3H1 cytodifferentiation. However, experiments which investigated the effect of the demethylating agent 5-azacytidine on BC3H1 cell differentiation strongly suggest that the muscle regulatory factors myogenin and protein Id may influence the transcriptional activity of both the SK and VSM α-actin genes. We also know from work presented earlier in this dissertation that SK and VSM α-actin gene expression is maximal when BC3H1 cells are cultured in N2 serum-free medium in contact with a maturation promoting biomatrix which is secreted by maturing BC3H1 cells, (referred to as SAM, Strauch et al. 1991). Although the observation that subconfluent BC3H1 cells can differentiate on SAM when cultured in N2 serum-free medium suggests that cell-cell contact is not absolutely required for initiation of the developmental program, cell-cell contact does reduce thymidine incorporation and may provide an essential trigger for cytodifferentiation in serum-containing medium.
As we have seen, serum reduction is itself not required for differentiation. Although reasonably well described at the cell phenomenological level, the molecular mechanisms by which these various epigenetic controls serve to regulate VSM α-actin transcription are largely unknown.

To investigate the influence of these epigenetic factors on VSM α-actin gene transcription, we assayed VSM α-actin promoter activity under several culture conditions by transiently transfecting BC3H1 myoblasts with plasmid expression vectors containing the bacterial gene for chloramphenicol acetyl transferase (CAT) placed under the control of various portions of the mouse VSM α-actin promoter. The 5' flanking and 5' UT regions of the mouse VSM α-actin gene, including the promoter region, as partially characterized by Min et al (1990) is diagrammed in Figure 3. Transient transfection studies have demonstrated that the first three CArG boxes upstream from the TATA box are required for "core promoter" activity in BC3H1 cells, while the fourth CArG element (box D) may serve as a cis-acting repressor element. The goal of the present study was to further identify regions of the VSM α-actin promoter which respond to epigenetic signals by using a transient transfection system and the set of truncated promoter/CAT reporter gene constructs which are diagrammed in Figure 4.

Our initial experiments were designed to determine the extent to which VSM α-actin promoter activity responds to simple, short-term cell-cell contact and more developed cell-substrate contacts. In order to test the effects of simple cell-cell contact independently,
with minimal interference from cell-substrate contacts which develop as myoblasts grow to confluence in culture and begin to secrete extracellular matrix proteins, we non-enzymatically harvested transfected BC3H1 myoblasts, then replated them at subconfluent densities. Additional non-transfected cells were added to some dishes of the replated transfected cells in order to increase cell density and provide short-term cell-cell contact stimulation. After 48 hours in DMEM containing 10% FBS, the "cell contact negative" cells (CC-) were not fully confluent while the "cell contact positive" cells (CC+) had just formed a confluent monolayer (Plate XV, panels B and C). As shown in Plate XV panel A, the CAT assays indicate that experimentally enhanced cell-cell contact apparently has no positive effect on promoter transcriptional activity in any construct. If anything, the result shown suggests that promoter activity may be somewhat reduced under conditions of high cell density prior to cytodifferentiation in serum-containing medium. If confirmed, this observation would be consistent with the Northern blot result discussed earlier which indicated that α-actin mRNA accumulation is reduced in confluent BC3H1 cells (refer to Plate VII).

In order to assess the effect of substrate composition and cell-substrate interactions on promoter activity, transfected cells were replated onto either fresh tissue culture plastic (TCP) dishes, or onto dishes coated with substrate attached macromolecules (SAM) synthesized by 6 day BC3H1 myocytes. The cells were seeded quite sparsely to minimize any cell-cell contacts, and were maintained in
Figure 3. The 5' Flanking Region of the Mouse Vascular Smooth Muscle $\alpha$-Actin Gene

The mouse VSM $\alpha$-actin gene, including the 5' flanking region, has recently been cloned and sequenced (Min et al. 1990). The upstream region contains evolutionarily conserved sequence motifs within a functional promoter. These motifs include CArG boxes (open boxes lettered A-D) and 'E' boxes (hatched boxes lettered E1-D6), both of which are known to serve as DNA binding domains for trans-acting transcriptional regulatory factors. The mouse CArG domains that are shared with the chicken VSM $\alpha$-actin promoter are identified by open boxes below the line in panel B; cross hatched CArG boxes below the line in panel B are not present in the chicken gene (Carroll et al. 1988). Mouse CArG boxes A, B, and C are shared with the human VSM $\alpha$-actin gene (Reddy et al. 1990). Diagram kindly provided by Dr. A. R. Strauch as adapted from Foster, Min, Foster, Stoflet, Getz, and Strauch (manuscript submitted).
Figure 4. Vascular Smooth Muscle α-Actin Promoter Region/CAT Reporter Gene Constructs

Transient transfections of BC3H1 myoblasts were performed using VSM α-actin promoter/CAT reporter gene fusion vectors, which were the generous gift of Dr. Michael Getz and are diagrammed above. Solid boxes represent CArG boxes, and hatched boxes represent the TATA box. Bent arrows indicate transcriptional start sites. The entire length of the upstream regulatory region is 1067 nucleotides. The constructs are labeled so that the start of transcription is at +1067 nucleotides downstream from the 5' end of the full length promoter. From top to bottom, constructs are abbreviated in the text using the shorthand notation P1 through P7. Diagram kindly provided by Dr. M. J. Getz, Mayo Clinic Research Foundation, Rochester, MN.
Transient transfections of subconfluent BC3H1 myoblasts with selected VSM α-actin promoter region/CAT reporter gene constructs (diagrammed in Figure 4 and indicated here by number in panel A) were performed by calcium phosphate precipitation. After a 24 hour recovery from transfection, the cells were non-enzymatically harvested and reseeded at subconfluent densities in DMEM 10% FBS (panel B). To half of the dishes, additional non-transfected cells were added to create extensive cell-cell contacts (panel C). After 48 hours, cells were harvested for CAT assays. The CAT assay results indicate that short-term cell-cell contacts do not increase the activity of the VSM α-actin promoter (panel A). Interestingly, the existence of cell-cell contacts results in slightly diminished CAT activity. Note that construct P4 has the highest level of activity in both high and low density preparations, since it lacks the fourth CArG box that is believed to function as a repressor element. Bar = 15 μM.
PLATE XV

CC-  CC+

A 1 2 3 4 7

B

1 2 3 4 7

C
To investigate the effect of myocyte-produced substrate attached macromolecules (SAM) on VSM α-actin promoter activity, subconfluent BC3H1 myoblasts were transiently transfected with the promoter/CAT constructs indicated and, after a 24 hour recovery, harvested and reseeded at subconfluent densities in N2 medium on either tissue culture plastic (TCP) or SAM prepared from 6 day N2 myocytes. Myoblasts were maintained in N2 medium for 8 days to permit full morphological differentiation on SAM, during which time the medium was not changed (panel C). Cells on TCP were largely quiescent in N2 (panel B). The CAT assay results suggest that SAM acts to induce VSM α-actin promoter activity in constructs P1 and P2, (and to a lesser extent in P3), and that construct P7 is not active on either TCP or SAM in serum-free N2 medium (panel A). Insufficient P4 extract existed to give any CAT activity in this experiment, but a similar experiment (Plate XVII) indicates that P4 activity is slightly inducible by SAM. Bar = 15 μM.
PLATE XVI

TCP

SAM

A 1 2 3 4 7 1 2 3 4 7

B

C
serum-free, hormone supplemented N2 medium. Under these conditions, cells on TCP are largely quiescent (Strauch et al. 91; and unpublished observations). As the CAT assay data shown in Plate XVI (panel A) reveals, SAM appeared to have a positive effect on the promoter activity of constructs P1, P2 and P3. The activity of constructs P4 and P7 was negligible both on TCP and on SAM. Initially we thought this result indicated that the high level of P4 activity observed in previous experiments shown in Plate XV (panel A) was totally serum-dependent, and we designed our subsequent experiment to not only reconfirm the results obtained using SAM but to identify putative serum response elements within the VSM α-actin promoter region. However, there was also the distinct possibility that noticeable cell detachment in dishes containing P4 transfected myoblasts resulted in insufficient cell extract to assay for CAT activity. Upon repeating the experiment, we found that SAM did have a detectable positive effect on the transcriptional activity of construct P4 (refer to Plate XVII). These data therefore suggest that cell-SAM interaction results in increased transcriptional activity of the VSM α-actin promoter, and that the truncated core promoter is also capable of responding to this development signal.

Our interest in the possible serum-inducibility of construct P4 came from the observation that the level of CAT activity in extracts of "cell contact negative" cells cultivated in DMEM 10% FBS was somewhat greater than the CAT activity detected in the extracts of cells at comparable density seeded onto tissue culture plastic in N2
serum-free medium (compare Plate XV, panel A and Plate XVI, panel A). This comparison appears to suggest that the P4 construct, and possibly the full length VSM \( \alpha \)-actin promoter as well, may be responsive to serum.

In order to address the question of promoter responsiveness to serum the following experiment was performed. Transfected BC3H1 myoblasts were reseeded at subconfluent densities on either TCP or SAM and cultured in N2 medium. After seven days, the majority of the cells on SAM had matured into spindle shaped myocytes while the cells on TCP retained their myoblastoid morphology (Plate XVII, panels B,C,F,G). On the seventh day, FBS was added to a final concentration of 10% to half of the dishes, and an equivalent volume of fresh N2 medium was added to the remaining dishes. Shape changes were evident after 12 hours in serum-containing medium: the spindle shaped myocytes were slowly beginning to modulate their phenotype to that of proliferating myoblasts. The cells to which FBS was added and the cells on N2 were harvested for cell extracts after 16 and 18 hours, respectively. As Plate XVII shows (panels A and D), this experiment not only reconfirms the positive inductive effect of SAM on promoter activity, but it also clearly shows that FBS increases the transcriptional activity of each promoter/CAT construct, including P3 and P7. This result is consistent with the Northern blot data showing that 6 day BC3H1 myocytes increase both non-muscle and muscle actin transcript levels after the addition of 10% FBS (Plate XVII E).
PLATE XVII. Transient Transfection Studies Investigating the Inducibility by Fetal Bovine Serum of the VSM \(\alpha\)-Actin Promoter

The transient transfection data presented in plate XV and XVI suggests that the VSM \(\alpha\)-actin promoter might be inducible by fetal bovine serum (FBS). To test this hypothesis, transfected myoblasts were non-enzymatically harvested, reseeded at subconfluent densities on either tissue culture plastic (TCP) or SAM, and maintained in serum-free N2 for 7 days, at which time FBS was added to a final concentration of 10\% to half the dishes, while the remaining dishes received a similar aliquot of fresh N2. After 16 hours the cells were harvested and extracts prepared for CAT assays. Prior to harvest, no gross differences were observable between cells grown on TCP and supplemented with N2 and cells grown on TCP and supplemented with FBS (compare panels B and C respectively); cells allowed to differentiate on SAM were typically spindle-shaped, but those maintained on SAM and supplemented with FBS were beginning to modulate their phenotype (compare panels F and G respectively). The level of CAT activity in cells maintained on TCP in serum-free N2 medium (panel A, constructs 1-7) increased for each construct following addition of serum (panel A, constructs 1'-7'). The level of
CAT activity in cells maintained on SAM in N2 (panel D, constructs 1-7) was slightly greater than that observed in cells maintained on TCP, confirming the positive inductive effect of SAM. However, following the addition of FBS the level of promoter activity increased for each construct (panel D, constructs 1'-7'), suggesting that serum is equally capable of inducing VSM α-actin promoter activity in quiescent myoblasts maintained on TCP in N2 or in differentiated myocytes on SAM in N2. The positive inductive effect of FBS on actin mRNA expression in fully-differentiated 6d N2 myocytes exposed to 10% FBS for 24 hours is evident from the Northern blot shown in panel E (lane 1 = 6d N2 MC, lane 2 = 6d N2 MC + FBS. Probed with pHMaA-1; upper band represents nonmuscle β- and γ-actin mRNAs while the lower band represents muscle α-actin mRNAs). Bar = 15 μM.
PLATE XVIII.  

Transient Co-transfection Experiments Investigating the Role of Myogenin in VSM $\alpha$-Actin Promoter Activation

Myogenin previously has been implicated in regulating the expression of skeletal muscle-specific genes (Wright et al. 1989; Olson 1990). In order to determine if myogenin also is involved in regulating smooth muscle $\alpha$-actin gene expression, transient co-transfections were performed using selected VSM $\alpha$-actin promoter constructs (P1, P2, and P4 as indicated by number), and the following three expression vectors: pMyogenin (M), containing a full length sense cDNA; pId (I), containing a full length sense cDNA; and pEMSV (E), an "empty" expression vector serving as a control for nonspecific stimulation of promoter activity. The results indicate that myogenin strongly induces VSM $\alpha$-actin transcriptional activity. Note that the P2 construct contains a single E box (refer to Figures 3 and 4), whereas the P1 construct contains six E boxes. Nevertheless, the P2 + pMyogenin co-transfection has a level of CAT activity that is equivalent to P1 + pMyogenin (compare 1M and 2M). This suggests that the first upstream E box is sufficient for full activation of the VSM $\alpha$-actin promoter by myogenin. The absence of any inhibitory effect by Id suggests that the low levels of VSM $\alpha$-actin promoter activity in myoblasts may be the result of serum-stimulation and/or specific myogenic activation by a non-HLH regulatory factor.
Clearly, the composition of the medium does influence core promoter activity, as it does activity of larger promoter constructs containing E-box elements. In addition, P3 appears to lack strong SAM responsiveness, unlike constructs P1 and P2, indicating that sequence elements located between -224 and -724 may mediate substrate response in the VSM α-actin promoter. These upstream sequences may contain DNA binding sites for positive trans-acting factors which are induced during cultivation on SAM in N2 and which serve to increase the transcriptional activity.

The 5-azaC experiments described earlier indicated that myogenin expression correlates with α-actin induction. This observation suggests that myogenin may serve as a transcriptional regulatory factor for VSM α-actin gene expression. Significantly, sequence analysis reveals that 6 "E boxes" are located in this upstream region. To test this hypothesis, subconfluent BC3H1 myoblasts were co-transfected with selected promoter/CAT vectors and either pMyogenin or pld expression vectors. Forty-eight hours after transfection the cells were harvested, cell extracts prepared, and assayed for CAT activity. The results clearly indicate that myogenin markedly increases the transcriptional activity of the VSM α-actin promoter in transfected myoblasts (Plate XVIII). Interestingly, the P2 construct contains only one E box but was induced by myogenin to a level equivalent if not slightly greater than the level of activity seen with P1 (P1 contains six E boxes). This suggests that the first E box just upstream from the fourth CArG element is perhaps the essential E box for activation by myogenin. Neither protein Id or pEMSV
(negative control) had any effect on VSM α-actin promoter activity in this subconfluent state. The absence of any inhibitory effect by Id indicates that the low level of VSM α-actin promoter activity in myoblasts may be the result of serum stimulation and not specific myogenic activation by an HLH regulatory factor. These results therefore suggest a mechanism by which both myogenin and serum promote the expression of VSM α-actin during BC3H1 myogenic cell differentiation.
DISCUSSION

From the transient transfection experiments described in this chapter, we can make the several observations regarding the activity of the VSM α-actin promoter under several culture conditions. First of all, it is readily apparent that in subconfluent myoblasts in serum-containing medium, the activity of the P4 construct is significantly greater than either the P1 or the P2 constructs, while the P3 construct shows little activity and the P7 construct shows no activity (refer to Plate XV). We know from work previously reported from our laboratory (Min et al. 1990) that a VSM α-actin promoter construct containing only two CArGs upstream from a TATA box is nonfunctional in BC3H1 cells and in nonmuscle AKR2B fibroblasts. As we have seen, however, the P4 construct containing 3 CArGs (A, B, and C CArGs) upstream from a TATA box is very active in BC3H1 cells at all stages and conditions. In addition, this construct is highly active in nonmuscle cells (A.R. Strauch, unpublished data), suggesting that the 3 CArG construct constitutes the non-selective "core" promoter required for VSM α-actin transcriptional activity. The fact that the P4 construct is active in nonmuscle cells suggests that the factors with
which it interacts in myoblasts are not restricted to muscle cell lineages. CArG elements have been shown to have the same consensus sixteen base core sequence as the serum response element (SRE), and CArG binding factor (CBF) has been shown to be identical to the Serum Response Factor (SRF) (Boxer et al. 1989). Thus, the high level of P4 activity in proliferating transfected myoblasts may be the result of positive trans-activation by CBF/SRF.

The activity of the P3 construct, which contains the fourth CArG element (D CArG) is minimal in myoblasts, suggesting that this fourth upstream CArG element functions in the transient transfection experimental assay system as a repressor of VSM $\alpha$-actin core promoter activity in proliferating myoblasts and perhaps nonmuscle cells (A.R. Strauch, unpublished data). This element may also somewhat restrict the activity of the P1 and P2 constructs under these conditions.

Compared to quiescent myoblasts maintained in N2 serum-free medium, the activity of all constructs (except for P7) is increased in SAM N2 myocytes. The activity of P1 and P2 in SAM myocytes is equivalent to P4, and although the activity of P3 is slightly induced in SAM myocytes, it is still significantly less than that detected for P1 and P2. This suggests that the region upstream from the D CArG box contains sequence elements which respond to transcriptional activating factors expressed in differentiating BC3H1 myocytes. The modest increase in P3 and P4 activity observed in SAM myocytes suggests that the 3' downstream CArG elements may also be able to respond to positive trans-acting factors produced by myocytes, a
phenomenon referred to by Walsh as "factor cross-binding" (Walsh 1989).

Co-transfection experiments with myogenin, protein Id, and selected VSM $\alpha$-actin promoter/CAT constructs indicate that myogenin is capable of increasing transcriptional activity of both P1 and P2 constructs in proliferating myoblasts. P1 contains six E box elements, while P2 has a single E box upstream from the D CArG element. This result suggests that the downstream E box is the critical E box for myogenin trans-activation. Given the high level of P4 activity in these myoblasts, it is not possible to determine from these preliminary experiments if the observed induction is the result of direct trans-activation by myogenin, or if myogenin is acting to induce expression of other myocyte-specific factors which bind to CArG elements. In this regard, the P4 construct is slightly (perhaps insignificantly) induced by myogenin co-transfection, implying that myogenin may have indirect as well as direct effects on VSM $\alpha$-actin promoter activity. Experiments are currently underway to determine if transcriptional activation by myogenin is dependent solely on the first upstream E box (E1). Finally, protein Id failed to inhibit transcriptional activity of either P1, P2, or P4 when co-transfected into proliferating BC3H1 myoblasts. This result indicates that the basal level of VSM $\alpha$-actin promoter activity observed in myoblasts transfected with P1 or P2, or the high level of activity seen with P4, is not dependent on transcriptional activation by an HLH myogenic factor such as myogenin. However, alternative explanations which might account for the apparent lack of Id-mediated inhibition, such as rapid
protein Id turn-over in the pre- to post-confluent BC3H1 myoblast, or the possibility that Id and myogenin do not interact, need to be investigated before we can draw any conclusions about the role of Id in BC3H1 myogenic differentiation.

In summary, the experimental observations from the transient transfection studies can be integrated to arrive at a model for transcriptional activation of the VSM α-actin gene during BC3H1 cytodifferentiation. In subconfluent, proliferating cells there is a low level of VSM α-actin expression. Transcription is activated at this stage by the presence of one or more non-HLH proteins inducible by serum, such as CArG binding factor (SRF), but transcription is kept to a low level in myoblasts by the inhibitory or 'governing' effect of the D CArG box. When serum levels are elevated, such as after changing the culture medium, and the number of cells which compete for the serum factors is low, then myoblasts may respond to the presence of serum by synthesizing CBF (SRF) and transcribing low but detectable levels of VSM α-actin mRNA. However, as the myoblasts continue to proliferate in culture and become more plentiful, the competition for the remaining serum factors becomes more intense, resulting in lower levels of SRF per cell and a reduced, barely detectable level of gene transcription. Upon attaining confluence, which we may define as the presence of extensive cell-cell contacts and the elaboration of SAM, cells are on the threshold of differentiation. There may be basal levels of VSM α-actin gene transcription at this stage even in the absence of serum factors, due to the presence of transcription factors which may participate in the formation of the transcription complex and
activation of transcription. Such factors, which presumably account for the relatively low level expression of P1 and P2 transcriptional activity seen in quiescent myoblasts, may be ubiquitous (e.g. Sp1), or specific to cells of a myogenic lineage (e.g. MAPF1, Walsh and Schimmel 1987).

Once myoblasts have begun to differentiate, (or if confluent myoblasts are re-fed with fresh DMEM 10% FBS), the VSM α-actin gene becomes highly active as indicated by nuclear run-on data showing pronounced upregulation at confluence. Northern blots of total RNA and nuclear run-on assays both indicate that myogenin is not expressed at this stage, suggesting that it is not myogenin which is primarily responsible for the increased level of VSM α-actin transcription in early myocytes. Rather, another novel transcriptional activating factor may be responsible for VSM α-actin promoter activation in early myocytes. Well-differentiated four day N2 myocytes are characterized by high levels of VSM α-actin gene transcription and mRNA accumulation. Myogenin is expressed by day 2 of differentiation, and could become involved in the late-stage transcriptional regulation of the VSM α-actin gene. The transfection data suggest that in myocytes, transcription factor binding occurs in both the CArG box region (serum-respose experiments) and upstream in the E-box region (myogenin expression experiments), indicating that perhaps transcription of the VSM α-actin gene in maturing myocytes, like the CA α-actin gene (Sartorelli et al. 1990), depends on the activity of multiple trans-acting factors which are targeted to different sites in the promoter.
This model is attractive in that it can account for every observation we have made pertaining to the regulation of VSM α-actin gene described in this dissertation. The model offers a reasonable explanation for the disparity between the absence of VSM α-actin mRNA and the presence of abundant nascent transcripts in "confluent" myoblasts. In addition, it offers an explanation for the ability of confluent BC3H1 cells to differentiate and express VSM α-actin in DMEM 10% FBS. The model also predicts that differentiation and expression of VSM α-actin in serum-containing medium would require the induction in late-stage cells of myogenic factors which may bind to regulatory sequences in the upstream promoter region. The transfection data suggests that myogenin can activate VSM α-actin transcription in early stage cells even in the presence of serum. However, full activation of the native gene by myogenin during differentiation in serum-containing medium may be slightly retarded by the competition of high levels of serum response factors for "accessory" transcription factors and regulatory sites within the promoter. This implies that differentiation in serum-containing medium might be somewhat delayed with respect to differentiation in serum-free medium, and this is in fact what we observe.

The transfection experiments as well as Northern blot studies indicate that the VSM α-actin gene is inducible by serum not only in quiescent myoblasts but also in differentiated SAM-myocytes. Under the conditions of these experiments, promoter activity and mRNA accumulation were measured within 24 hours after addition of serum to a final concentration of 10%. To further understand the serum
inducibility of the VSM $\alpha$-actin gene, a time course study will be required to determine the onset and duration of serum-induced transcriptional activation in myoblasts and myocytes. Previous studies have indicated that while sparsely seeded BC3H1 cells will modulate their phenotype in response to serum and alter their pattern of $\alpha$-actin expression, fully-differentiated confluent myocytes are more refractory to serum stimulation and remain relatively well differentiated (Spizz et al. 1986; A.R. Strauch, unpublished observations). This result serves to emphasize the "plastic" nature of the VSM $\alpha$-actin gene and its ability to respond to microenvironmental inputs.

The data presented also suggest that the regulation of VSM $\alpha$-actin expression during BC3H1 cell differentiation is under negative control. In Chapter II data were presented suggesting that native expression of protein Id in myoblasts may prevent the onset of differentiation. Additionally, results from these transient transfection studies indicate that expression of the VSM $\alpha$-actin gene in myoblasts and nonmuscle cells is to some extent repressed by the action of the fourth CArG element. Studies are currently underway in our laboratory to further elucidate the role of protein Id and to identify nuclear factors which may bind to the D CArG element in hopes of further characterizing this interesting aspect of VSM $\alpha$-actin expression during myogenesis.

Finally, the data presented in this chapter indicate that the VSM $\alpha$-actin full-length promoter construct responds to microenvironmental inputs in a manner that is consistent with the
response of the native gene to similar inputs as documented in this study and elsewhere (Strauch et al. 1991). This suggests that transient transfections of BC3H1 myogenic cells with VSM $\alpha$-actin promoter/CAT constructs can provide meaningful information about the function of the VSM $\alpha$-actin promoter region. Although useful for obtaining preliminary results such as those reported here, in many respects the utility of the transient transfection assay system is limited. Plasmid can be lost during cell growth and division, and through cell attrition in culture, leading to potentially misleading results, (as was observed for the P4 transfected cells in the SAM vs TCP experiment). In addition, transient transfection limits the length of time transfected cells can be maintained in culture, especially under conditions in which cells proliferate. Assaying equivalent amounts of total protein for each transfected cell extract in a given experiment is one way to correct for varying cell numbers among different transfected cell populations, but a more sophisticated and informative means of simultaneously correcting for varying cell numbers and transfection efficiency would have been to include in the transfections a second reporter gene construct whose activity would serve as an internal control e.g. a $\beta$-galactosidase reporter gene under the control of a constitutive promoter such as the metallothionein promoter. Data could then be normalized with respect to the activity of the reporter gene, or amounts of extract containing equivalent activity of the control reporter gene could be assayed for CAT activity.

Even with elaborate controls monitoring transfection efficiency, however, in a transient transfection system there is no means of
guaranteeing that all cells will receive equivalent amounts of plasmid. While not likely to be a significant source of error, local microenvironmental differences between transfected and nontransfected cells may also skew the results obtained. For these and other reasons, the ideal method of determining activity of heterologous promoters in transfected cells is to establish stably transfected cell lines. Use of clonally-selected, stably transfected cells insures that all cells in the culture are transfected and possess the same copy number of the introduced gene. There are no practical limitations on the length of time these cells can be maintained under a given set of experimental conditions. Other members of our laboratory recently established a "library" of stably transfected BC3H1 cells using the different VSM α-actin promoter constructs under G418 (neomycin) antibiotic selection. These cell lines will be used to obtain definitive information regarding the effect of microenvironmental influences and cellular adaptations on VSM α-actin promoter activity.
GENERAL DISCUSSION

The experiments described in this dissertation were designed to improve our understanding of the regulation of α-actin isoform expression during mammalian myogenic cell differentiation. We have used the BC3H1 myogenic cell line as a model system for studying the epigenetic mechanisms which control actin isoform expression during cytodifferentiation. Nonmuscle β- and γ-actins are the predominant actin isoforms expressed in proliferating undifferentiated BC3H1 myoblasts, while post-confluent differentiated myocytes in serum-free, hormone-supplemented medium down-regulate nonmuscle actin expression and up-regulate VSM α-actin expression. We have shown that BC3H1 myocytes also synthesize SK α-actin, and that expression of SK α-actin mRNA and protein during BC3H1 cell differentiation occurs after the observed upregulation of VSM α-actin.

Nuclear run-on assays have shown that the regulation of α-actin expression in BC3H1 cells occurs, at least in part, at the level of gene transcription. In an effort to characterize some of the transcriptional control mechanisms regulating α-actin expression, BC3H1 myoblasts were cultured in the presence of the hypomethylating agent 5-azaC. These experiments, along with the results of Southern blot analysis of
α-actin gene methylation status in BC3H1 myoblasts and myocytes, suggest that α-actin transcription is probably not directly regulated by cytosine methylation. However, these experiments do suggest that the myogenic regulatory factors myogenin and protein Id may play a role in controlling α-actin expression during BC3H1 cytodifferentiation. In order to test this hypothesis, transient transfection experiments were performed using VSM α-actin promoter region/CAT reporter gene plasmid constructs. Results from these experiments indicate that myogenin is involved in VSM α-actin transcriptional activation. To my knowledge, this is the first report of transcriptional activation of the VSM α-actin promoter by an HLH myogenic regulatory factor.

We have also demonstrated that the BC3H1 differentiation program, as reflected by cellular morphology, by the accumulation of α-actin mRNA, and by myogenin expression, is affected by the cellular microenvironment. Transient transfection studies indicate that the VSM α-actin promoter region contains DNA sequences which may mediate substrate- and culture medium-dependent changes in gene transcription, possibly by binding trans-acting myogenic regulatory factors. Taken together, these results suggest that α-actin expression during BC3H1 cytodifferentiation is more closely linked to extrinsic factors than to intrinsic, genetically programmed developmental signals.

Clearly, further experimentation is required in order to confirm and extend several of the observations summarized above. For example, to confirm the apparent transcriptional regulation of the protein Id gene by cytosine methylation, nuclear run-on experiments...
should be performed to document the suspected correlation between the change in Id gene methylation status and Id gene transcription. DNase I sensitivity assays would be of additional benefit in understanding not only the potential role of methylation in Id gene regulation, but also the apparent transcriptional regulation of the α-actin genes during BC3H1 cytodifferentiation. Sensitivity to digestion with DNase I has previously been shown to correlate with gene transcriptional activity (Weintraub and Groudine 1976).

The roles of myogenin and Id in α-actin gene expression need to be further investigated. A transient transfection experiment just completed (data not shown) complements the experimental result depicted in Plate XVIII and, taken together with the earlier data, suggests that 'E' box number 1 (E1, at approximately -330 bases upstream from the transcriptional start site) is required for maximal activation of VSM α-actin promoter activity by myogenin. Whether or not E1 alone, without associated downstream CArG elements, is sufficient for transcriptional activation of the VSM α-actin gene could be addressed by preparing additional deletion mutants of the promoter region. In addition, DNase I protection/methylation interference assays and/or gel band retardation assays need to be performed in order to determine if in fact myogenin is binding to the VSM α-actin promoter, or whether the stimulatory effect observed upon myogenin co-transfection is due to induction of other myogenic regulatory factors which bind to novel sequences within the promoter region. Work attempting to identify CArG binding factors within
BC3H1 myoblast and myocyte nuclei is already in progress in the laboratory.

In addition to understanding the role of myogenin in promoting BC3H1 differentiation, we need to understand the apparent role of Id in inhibiting differentiation. It is known that Id binds to both E12 and myoD, thereby preventing myoD from binding to E box sequence elements as a myoD-myoD homodimer or as an E12-myoD heterodimer. The ability of Id to interact with myogenin has not yet been investigated. One approach to investigating this question would be to employ a gel band retardation assay to determine if Id can inhibit myogenin binding to VSM α-actin promoter sequences, (if myogenin/VSM promoter binding is confirmed). Another approach would be to co-transfect myogenin and Id expression vectors into BC3H1 myoblasts along with VSM α-actin promoter/ CAT reporter gene vectors and to observe if Id can inhibit the transcriptional activating effect of myogenin.

We have shown that α-actin expression is affected by the cellular microenvironment. Precise identification of cell-cell contact, SAM, and serum responsive regions within the VSM α-actin promoter region will require the use of stably transfected cells. Stable transfectants using VSM α-actin promoter/human growth hormone (hGH) reporter gene constructs have recently been produced in our laboratory, and will permit us to analyze VSM α-actin transcriptional activity under a variety of cell-cell and cell-substrate contact conditions. Stable transfection would also be the ideal way to perform experiments using antisense myogenin and antisense Id constructs to
explore the role of these two myogenic factors in transmitting signals from the microenvironment to the VSM α-actin promoter. Finally, we need to undertake a more thorough characterization of the important transition from proliferating myoblast to differentiating myoblast. The state of "confluence" has been difficult to define and reproduce in practice, and it needs to be better understood so that we can more accurately describe the events which lead to the onset of BC3H1 myogenic cell differentiation and the expression of VSM and SK α-isoactins.

A great deal of work needs to be done before we can describe in detail the mechanisms which govern α-actin expression during myogenesis. The data that I have presented in this dissertation have given us some insight into the nature of α-actin gene regulation in BC3H1 cells, but like most experimental data these results raise more questions than they answer and the additional experiments outlined above go only part of the way towards answering those questions. Despite these caveats, however, it is tempting to speculate what these data might tell us about not only how α-actin expression is regulated during myogenesis but also why it is regulated in this manner, i.e. why is myogenesis characterized by actin isoform switching?

Leavitt and co-workers (Leavitt et al. 1987a,b) demonstrated that in normal diploid human fibroblasts transfected with a mutant nonmuscle β-actin the total amount of actin protein synthesized remained constant. To "compensate" for the increased expression of β-actin by the exogenous mutant gene, levels of endogenous wild type
β- and γ-actin mRNA were reduced. In addition, immunocytochemical analysis revealed that the mutant actin was preferentially localized within the actin-containing structure in the cell. Specifically, the mutant β-actin accumulated in stress fibers, but was not detected in the actin-rich perinuclear network (Leavitt et al. 1987a). This observation led to the hypothesis that these cells, indeed perhaps all cells, employ an autoregulatory mechanism to maintain an optimal steady-state concentration of actin, and that actin isoforms do segregate according to function within the cell.

In a recent report, Gunning and co-workers (Wade et al. 1990) presented a detailed examination of contractile protein gene family mRNA pool sizes during myogenesis, and arrived at the conclusion that each gene was expressed in a unique pattern with no apparent coordination of expression between the gene families. However, total mRNA levels within gene families remained fairly constant throughout muscle differentiation. In light of other reports in the literature, including a previous report examining tropomyosin expression during myogenesis (Gunning et al. 1990), the authors interpreted these data as being supportive of an "isoform competition/autoregulatory compensation" feedback mechanism operating at the level of mRNA accumulation within each gene family. A similar conclusion was reached by Sutherland et al. (1991), who presented data indicating that each contractile protein gene has unique determinants of mRNA accumulation, and that myoblasts are not intrinsically programmed to coordinately express contractile protein isoforms in different gene families. The model which evolves from these observations can be
broken down into three parts. First, the model predicts that the mRNA level for a particular contractile protein gene family (e.g. actin) is fixed at a steady state level. Secondly, the percent contribution of a particular isoform (e.g. VSM α-actin) to the total mRNA pool for that gene family is dependent on the functional demand for that isoform within the cell. Finally, the commitment of a muscle cell to a "coordinated isoform phenotype" occurs in response to extrinsic factors. This model, then, views muscle differentiation as a dynamic process in which the cell "customizes" its contractile protein isoform profile in response to microenvironmental and physiological considerations. Of course, this model presupposes that the various contractile protein isoforms do indeed have functional differences. Exactly what the isoform functional differences are, how muscle cells utilize the different isoforms, and how the accumulation of mRNA is autoregulated, (i.e. at the level of transcription, post-transcription, or both), await further investigation. The data presented in this dissertation is, however, supportive of such a model in that we observe subtle changes in α-actin mRNA accumulation in response to different microenvironmental conditions. The observation that myogenin is involved in controlling VSM α-actin promoter activity also suggests one way in which α-actin gene transcription during myogenesis can be regulated in response to microenvironmental signals.
APPENDIX

The following diagrams represent schematic drawings of restriction maps for the various plasmid cDNA and genomic clones used during the course of this study. Maps are not drawn precisely to scale. References (when available) are given for further information about each clone.
pJ3' consists of a 175 bp Pst I fragment from the mouse skeletal α-actin 3' untranslated region inserted into pUC 8. pJ3' was isolated by Sandra B. Sharp from a cDNA library constructed by Robert J. LaPolla, California Institute of Technology. pJ3' is a skeletal α-actin specific probe.
Figure 7. pHMcA-3'UT-DB.

pHMcA-3'UT-DB consists of a 177 bp Dde I-BstN I fragment from the human cardiac α-actin 3' untranslated region inserted into the Eco RI site of pBR322 (Gunning et al. 1984b). pHMcA-3'UT-DB is a cardiac α-actin specific probe.
p12b represents an approximately 3.5 kB 5'-flanking region/exon 1/intron 1 clone of the mouse VSM α-actin gene inserted into pUC 119 (Min et al. 1990). p12b is a VSM α-actin specific probe, since exon 1 encodes the gene-specific 5'UT region.
Figure 9. **Mouse VSM α-Actin 3'UT cDNA Clone**

This clone represents the Dde I 160 bp fragment from the 3' untranslated region of the mouse vascular smooth muscle alpha-actin gene inserted into the Eco R1 site of pBluescript™ (Stratagene, LaJolla, CA). This 3'UT clone is a VSM α-actin specific probe, and was constructed by Sandra B. Sharp from a BC3H1 myocyte cDNA library prepared by Bonhong Min, The Ohio State University. MCS = Multiple Cloning Site.
Figure 10. **pEMSVscribe α2** (pEMSV)

pEMSV represents a eukaryotic expression vector developed by Harold Weintraub and co-workers at the Fred Hutchinson Cancer Research Center, Seattle, WA. It consists of a modified Bluescribe™ vector (Stratagene) into which a Long Terminal Repeat (LTR) from the Maloney Sarcoma Virus and an SV40 poly A (+) sequence have been inserted. The unique *Eco RI* site permits insertion of DNA sequences into the expression vector between the LTR and the poly A(+) site (Davis et al. 1987). MCS = Multiple Cloning Site.
Figure 11. pEMSV688SK

pEMSV688SK represents a 688 bp fragment containing both exon 1 and intron 1 and part of the 5'-flanking region of the mouse SK α-actin gene inserted into an Eco R1 - Bam H1 site of pEMSV. The 688 bp fragment was subcloned out of the parent M13 based clone "18-5" (Sharp et al. 1989, Hu et al. 1986) and placed into pEMSV to facilitate plasmid amplification.
Figure 12. Myogenin (pMyogenin)

This clone represents a full length (approximately 1.5 kB) myogenin cDNA inserted into the unique Eco R1 site of pEMSV in the sense orientation (Wright et al. 1989). MCS = Multiple Cloning Site.
Figure 13. Protein Id (pld)

pld represents a full length (approximately 900 bp) cDNA of protein Id inserted into the unique Eco RI site of pEMSV (Benezra et al. 1990).


